Effects of Purvalanol A on Imatinib-Sensitive and -Insensitive Chronic Myeloid Leukaemia Cell Lines

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

Panrawee Phoomvuthisarn, DVM, MSc

September 2015

I declare that this thesis entitled:

“Effects of Purvalanol A on

Imatinib-Sensitive and -Insensitive

Chronic Myeloid Leukaemia Cell Lines”

is entirely my own work

Candidate: Panrawee Phoomvuthisarn, DVM, MSc

Supervisors: Professor S.W. Edwards

Faculty of Health and Life Sciences

University of Liverpool

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I have found passion in science and this will light my way from now on. I would like to simply say ‘this is the best decision of my life, thank you very much indeed’.

ABSTRACT

Objectives: This project aimed to investigate effects of purvalanol A on imatinib-sensitive and -insensitive chronic myeloid leukaemia (CML) cell lines, and normal white blood cells.

Methods: Imatinib-sensitive (LAMA-84) and –insensitive (KCL-22) CML cell lines and human neutrophils and PBMCs were incubated with imatinib, and purvalanol A. Cell apoptosis was measured using flow cytometry and the expression of proteins was measured by Western blotting. Mcl-1 mRNA expression was quantified using real-time PCR.

Results: The CDK2 inhibitor, purvalanol A, induced cell death in both CML cell lines. In the KCL-22 cell line, purvalanol A treatment resulted in a rapid (≤1 h) decrease in levels of the anti-apoptotic protein, Mcl-1. This decrease in Mcl-1 was mediated by a decrease in mRNA and a marked acceleration in the rate of turnover of the protein (half-life decreased from ~3 h in control cells to ~1.5 h in drug-treated cells). Purvalanol A also induced cell death in LAMA-84 cell line, although it was not as potent and cell death occurred in the absence of a decrease in Mcl-1, suggesting different signalling pathways in these two lines and hence different capacity to alter Mcl-1 stability. Furthermore, purvalanol A also increased Mcl-1 turnover rate and phosphorylated p38-MAPK activity in neutrophils and in KCL-22 cells. In addition, the p38 MAPK inhibitor, BIRB796, decreased apoptosis and prevented the decrease in Mcl-1 expression induced by purvalanol A in both CML cell lines and neutrophils.

Conclusions: The development of drug resistance in patients with CML results in a continuing need to develop new ways to treat patients that become refractory to tyrosine kinase inhibitors. The results of this study indicate that purvalanol A might provide an adjunct to TKIs in the therapy of CML. This study shows that the effects of purvalanol A on induction of cell death in KCL-22 cell line are independent of the effects of this drug on cell cycle kinetics, but instead point to a direct or indirect effect on post-translational modifications of Mcl-1 that enhance its turnover rate.

PUBLICATION AND PRESENTATION

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ABBREVIATIONS

ABC	ATP binding cassette
ABCB1	Polyglycoprotein multidrug resistance 1
ABCC1	Multidrug resistance-associated proteins 1
ABCG2	Breast cancer resistance protein
ABD	Actin-binding domain
ABL	Abelson
Akt	Protein kinase B
ANOVA	Analysis of variance
AP	Accelerated phase
Apaf-1	Apoptotic protease-activating factor-1
APS	Ammonium persulphate
ATP	Adenosine triphosphate
Bad	B-cell leukaemia-2 associated death promoter protein
Bak	B-cell leukaemia-2 homologous antagonist/killer protein
Bax	B-cell leukaemia-2 associated protein-X
BCA	Bicinchoninic acid
Bcl-2	B-cell leukaemia-2 protein
Bcl-w	B-cell leukaemia-w protein
Bcl-X_L	B-cell lymphoma-extra large protein
BCR	Break point cluster region
Bfl-1/A1	B-cell leukaemia-2 related protein A1
BH	B-cell leukaemia-2 homology domain

Bid	BH3 interacting domain death antagonist protein
Bik	Bcl-2 interacting killer
Bim	B-cell leukaemia-2 like protein-11
Bmf	Bcl-2 modifying factor
Bok	Bcl-2 related ovarian killer
BP	Blast phase
BSA	Bovine serum albumin
CAK	CDK-activating kinase
Casp	Caspase cleavage
CC	Coiled/coil oligomerisation
CLL	Chronic Lymphocytic Leukaemia
CDK	Cyclin dependent kinase
cDNA	Complementary DNA
CGL	Chronic granulocytic leukaemia
CHX	Cycloheximide
cIAP	Cellular inhibitor of apoptosis
c-KIT	Stem-cell growth factor receptor
CKIs	Cyclin dependent kinase inhibitors
CLPs	Chronic lymphoid progenitors
CML	Chronic myeloid leukaemia
CMPS	Chronic myeloid progenitors
CORE	Committee for research ethics
CP	Chronic phase
D	Aspartate

DEPC	Diethylpyrocarbonate
DH	Dbl/CDC24 guanine nucleotide exchange factor homology
DIABLO	Direct IAP binding protein with low pI
DISC	Death-inducing signalling complex
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dsDNA	double stranded DNA
DTT	Dithiothreitol
E	Glutamate
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetracetic acid
ER	Endoplasmic reticulum
Erk	Extracellular signal-regulated kinase
F	Phenylalanine
FADD	Fas-associated death domain-containing protein
FasL	Fas ligand
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide-*4*-(trifluoromethoxy) phenylhydrazone
FDA	Food and Drug Administration
FISH	Fluorescence *in situ* hybridisation
FITC	Fluorescein isothiocyanate
G	Glutamate
GAB2	GRB-2 associating binding protein 2

GDP	Guanidine diphosphate
GM-CSF	Granulocyte macrophage colony stimulating factor
GMPs	Granulocyte/macrophage progenitors
GRB2	Growth factor Receptor-Bound protein 2
GSK3	Glycogen synthase kinase-3
GTP	Guanidine triphosphate
H	Histidine
H_2O_2	Hydrogen peroxide
HBSS	Hanks balanced salt solution
HRK	Harakiri
HRP	Horseradish peroxidase
HSCs	Haematopoietic stem cells
I	Isoleucine
IAPs	Inhibitors of apoptosis proteins
IFN-γ	Interferon-γ
IgG	Immunoglobulin G
IL	Interleukin
JAK	Janus kinase
JNK	C-Jun N-terminal kinase
kDa	kilo Dalton
$KHCO_3$	Potassium hydrogen carbonate
M	Methionine
MAPK	Mitogen-activated protein kinase
Mcl-1	Myeloid cell leukaemia 1

MEG	Megakaryocyte
MEK	Mitogen-activated protein kinase
MEPs	Progenitors of red blood cells and megakaryocytes
MHC	Major histocompatibility complex
MOMP	Mitochondrial outer membrane permeabilization
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor κB
NH_4CL	Ammonium chloride
NK cells	Natural Killer cells
NLS	Nuclear localization signals
Oct-1	Organic cation transporter-1
PBMCs	Polymorphonuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor
PDK-1	Phosphoinositide-dependent kinase-1
PEST	Proline-glutamic acid-serine-threonine
Ph	Philadelphia chromosome
PH	homology
Phos	Phosphorylation
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-Kinase
PP	Proline-rich SH3 binding sites
PUMA	p53 up-regulated modulator of apoptosis

PVDF	Polyvinylidene fluoride
qPCR	Qualitative polymerase chain reaction
RAF	Raf kinase
Rb	Retinoblastoma protein
RBC	Red blood cell
RPMI	Roswell park memorial institute
RT-PCR	Reverse transcriptase-polymerase chain reaction
S	Synthetic
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SFKs	Src family kinases
SH	Src homology
siRNA	Small interfering ribonucleic acid
Smac	Mitochondria-derived activator of caspases
SOS	Son of sevenless
STAT	Signal transducer and activator of transcription
T	Threonine
tBID	truncated BID
TEMED	Tetramethylethylenediamine
TKIs	Tyrosine kinase inhibitors
TNF-α	Tumour necrosis factor-α
TRADD	TNFR-associated death domain-containing proteins
Ub	Ubiquitination
V	Valine

WBC	White blood cell
WHO	World health organisation
Y	Tyrosine
Y-K	Tyrosine kinase

CHAPTER 1: INTRODUCTION

1.1 Chronic Myeloid Leukaemia

Chronic Myeloid Leukaemia (CML), also known as chronic granulocytic leukaemia (CGL), is a cancer of white blood cells. It is characterised by the unregulated growth of predominantly myeloid cells in the bone marrow and accumulation of these cells in the blood. This results from the BCR-ABL gene products in haematopoietic stem cells (HSCs), which differentiate into common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). CMPs may then differentiate into granulocyte/macrophage progenitors (GMPs) and progenitors of red blood cells and megakaryocytes (MEPs), before differentiating into many types of circulating cells, such as granulocytes (G), macrophages (M), megakaryocytes (MEG) and red blood cells (RBC) (Ren, 2005), (Figure 1.1). Proliferation of mature granulocytes such as neutrophils, eosinophils, and basophils, along with their precursors, results in many of the clinical signs found in CML patients.

1.1.1 Incidence

CML accounts for approximately 15-20 % of all newly diagnosed adult leukaemia cases and 14 % of leukaemia overall (including the paediatric population) (Jemal et al., 2010). The incidence rate is higher in men compared to woman (Rohrbacher and Hasford, 2009). The incidence and the age of onset of this disease in Western countries appears to be higher compared to Asian countries, as described in Table 1.1.

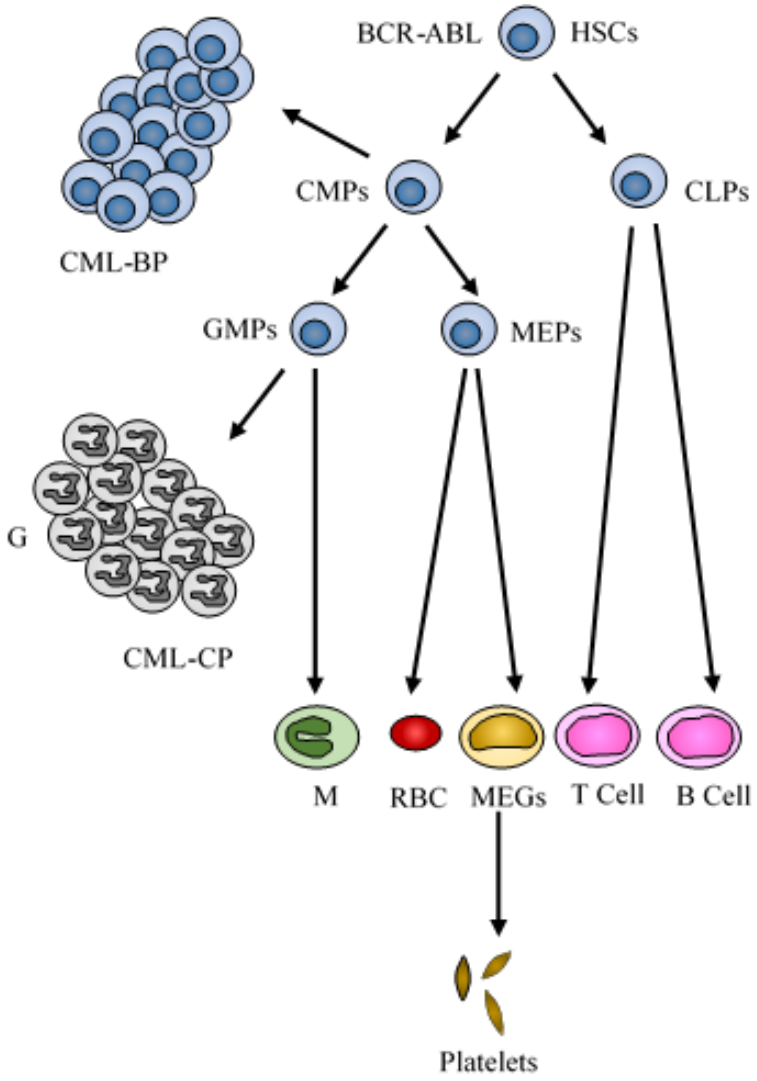

Figure 1.1 The development of chronic myelogenous leukaemia In CML chronic phase (CML-CP), there are high numbers of granulocytes circulating in the blood. CML can progress from the chronic phase into blast phase (CML-BP). (HSCs = hematopoietic stem cells; CMPs = common myeloid progenitors; CLPs = common lymphoid progenitors; GMPs = granulocytes (G) and macrophage (M) progenitors (GMPs); MEPs = megakaryocytes and erythrocytes progenitors; RBC = red blood cells; MEGs = and megakaryocyte) (Redrawn from (Ren, 2005)).

Table 1.1 Characteristics of the CML populations of Western and Asian countries.

Country/region	Source	Annual incidence ($100,000^{-1}$)	Median Age of diagnosis
United Kingdom	Haematological Malignancy Research Network (Smith et al., 2014)	0.9	59 y
USA	North American Association of Central Cancer Registries (Siegel et al., 2014)	1.48	65 y
China	Local Surveys (Au et al., 2009)	0.39-0.55	45-50 y
Thailand	Nine leading University hospitals (Au et al., 2009)	0.5	36-38 y

1.1.2 Diagnosis and Laboratory Findings

This disease is diagnosed on routine physical examination and blood tests. 20-40 % of asymptomatic patients are diagnosed to have CML by a persistence of leucocytosis (median white blood cell (WBC) count ~100,000/μL) with a population of neutrophils in different developmental stages, and high levels of segmented neutrophils and myelocytes (Savage et al., 1997, Cotta and Bueso-Ramos, 2007, Spiers et al., 1977).

The presence of the Ph chromosome is one of the characteristics of CML. It can be detected by cytogenetic, fluorescence *in situ* hybridisation (FISH), and polymerase chain reaction (PCR) (Jabbour et al., 2008, Kantarjian et al., 2008, Schoch et al., 2002). FISH analysis, based on the

hybridisation of fluorescent genomic probes specific to BCR and ABL genes is useful to detect the Ph chromosome, while PCR assays, based on amplification of the region around the splice junctions between BCR and ABL gene are also used. Both reverse transcriptase-polymerase chain reaction (RT-PCR) and qualitative polymerase chain reaction (qPCR) are suitable for CML diagnosis, while qPCR is ideal for monitoring residual disease (Vardiman, 2009, Stentoft et al., 2001, Lowenberg, 2003).

1.1.3 Clinical Manifestations and Staging

CML disease has 3 phases: (1) chronic phase (CP); (2) accelerated phase (AP); (3) blast phase (BP). In the first 3-5 years period after disease onset, CML patients are in the CP, which is characterised by high white blood cell counts, splenomegaly and extra medullary haematopoiesis. CP has common clinical signs of leucocytosis, fatigue, gout, weight loss, malaise, and left upper quadrant-pain, which results from anaemia and splenomegaly. Rare manifestations, which may include bleeding, thrombosis, and retinal haemorrhage are also found in some cases. Untreated patients will progress into the AP, but approximately 20 % of the patients will enter into BP without AP (Jabbour and Kantarjian, 2014).

AP is known as a progressive phase. According to the World Health Organisation (WHO), AP is characterized by persistence of elevated WBC counts (>100,000/μL), splenomegaly, persistent thrombosis (platelet count over than 1,000,000/μL), persistent thrombocytopenia (platelet count less than 100,000 / μL), and increased basophils (>20 % in peripheral blood) and myeloblasts (>10-19 % in peripheral blood) (Vardiman, 2010).

BP is a fatal acute phase. The immature myeloid cells (blast cells) are expanded rapidly due to rapid disease progress (Jaiswal et al., 2003). BP is characterized by persistence of blast cells (>20 % of the peripheral blood leukocytes) and a medullary infiltration by blast cells (Vardiman, 2010). In this phase, most of the patients suffer from the accumulation of blast cells, resulting in an increased susceptibility to infections and a shortage of red blood cells and platelets, two cell types that are derived from the same myeloid progenitor.

1.1.4 Pathophysiology

CML is associated with expression of the BCR/ABL fusion gene which encodes a constitutively-active protein tyrosine kinase, leading to a deregulation of tyrosine kinase activity in cells (Deininger et al., 2000). The downstream signalling events activated by this kinase activity result in the CML phenotype (Quintas-Cardama and Cortes, 2009). A variety of signalling pathways are affected by the uncontrolled kinase activity of BCR-ABL and these are usually involved in pathways associated with cell proliferation, control of apoptosis, differentiation and adhesion, all of which are implicated in CML pathology.

The role of BCR-ABL in CML was confirmed by studies in murine models (Daley et al., 1990, Kelliher et al., 1990, Elefanty et al., 1990). Mice that were induced to be express endogenous BCR-ABL via retrovirus-transduced bone marrow transplantation, developed CML-like myeloproliferative disorder (Daley et al., 1990, Lowenberg, 2003). This experiment confirmed that BCR-ABL is required to induce leukaemogenesis *in vivo*.

1.1.4.1 Philadelphia Chromosome

CML results from a single genetic abnormality leading to haematological malignancy. The chromosomal abnormality is known as the Philadelphia (Ph) chromosome, which is found in over 90 % of all cases diagnosed as CML (Shepherd et al., 1995). The Ph chromosome is the result of a fusion caused by the reciprocal translocation of the Abelson (ABL) tyrosine kinase gene on the 3' end of chromosome 9 and the break-point cluster region (BCR) gene on the 5' end of chromosome 22 (Figure 1.2) (Rowley, 1973). This translocation results in the formation of the BCR-ABL gene, which encodes an abnormal chimeric fusion protein, which is a constitutively active protein tyrosine kinase (Quintas-Cardama and Cortes, 2006). Three different types of fusion protein are formed depending on the breakpoint of BCR gene. The most common is the 210-kDa fusion protein, but 230-kDa and 190-kDa fusion proteins are also found (Clark et al., 1987, Fainstein et al., 1987, Deininger et al., 2000).

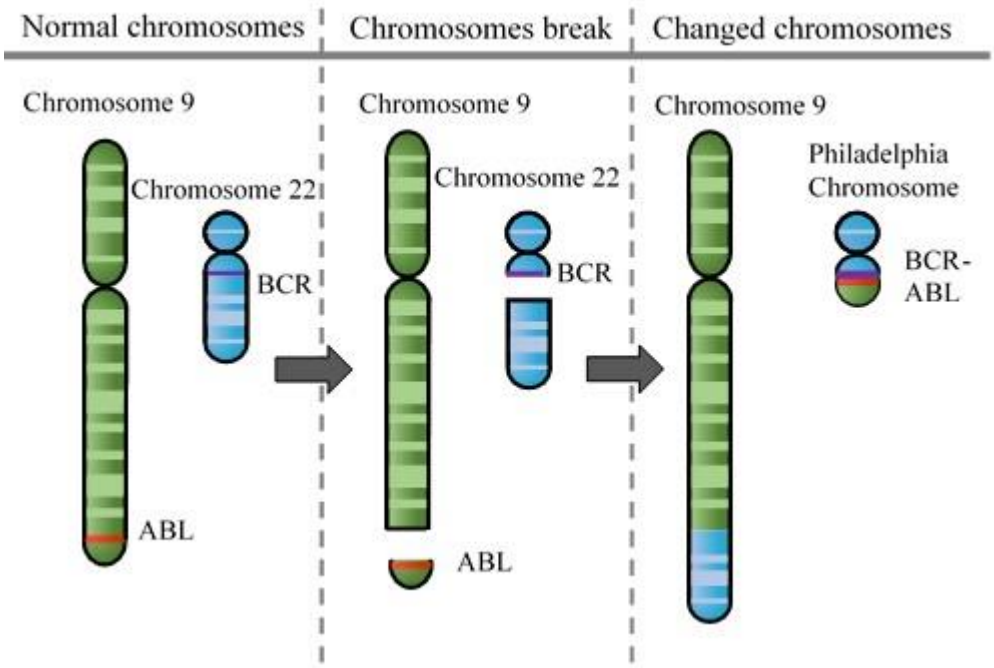

Figure 1.2 Philadelphia Chromosomes In normal chromosomes, the ABL and BCR genes are located on chromosomes 9 and 22, respectively. The BCR-ABL fusion gene is formed on the derivative chromosome 22 due to the (9;22) translocation. (Redrawn from (Lydon, 2009)).

1.1.4.2 Anatomy and auto-regulation of BCR-ABL protein

ABL is the human homologue of the viral abl (v-*abl*) oncogene carried by the Abelson murine leukaemia virus (A-MuLV) (Abelson and Rabstein, 1970). This gene encodes a non-receptor tyrosine kinase that is expressed in many tissues (Laneuville, 1995). ABL protein (145 kDa) is distributed in both the cytoplasmic and nuclear compartments of cells and normally moves between these two compartments in order to transduce signals from cell surface growth factor receptors for regulating cytoskeleton structure (Woodring et al., 2003). In addition, the ABL gene is involved in cell cycle regulation in the cellular response to genotoxic stress (Kipreos and Wang, 1990, Sawyers et al., 1994, Yuan et al., 1999).

The ABL central core consists of a tyrosine kinase, Src-Homology-2 (SH2) and a SH3 domain. Its last exonic region contains 4 proline-rich PP motifs which serve as binding sites of the SH3 domains of other proteins (Hantschel and Superti-Furga, 2004, Smith et al., 1999, Cohen et al., 1995). Toward the 3' end of the gene, DNA-binding, nuclear localisation signals and actin-binding motifs are also found (Kipreos and Wang, 1990).

The BCR gene encodes a 160-kDa protein, which, like the ABL protein, is ubiquitously expressed. The only substrate of this kinase that has been discovered so far is Bap-1, a member of 14-3-3 family of proteins (Reuther et al., 1994). It is also a signalling protein that contains multiple modular domains, such as a coiled/coil oligomerisation (CC) domain, serine/threonine kinase (ST-K) domain, pleckstrin homology (PH) domain, and Dbl/CDC24 guanine nucleotide exchange factor homology (DH) domain. The CC domain plays a role in dimer formation (McWhirter et al., 1993), while PH and DH domains stimulate the exchange of guanosine triphosphate (GTP) to guanosine diphosphate (GDP) on Rho guanine exchange factors (Denhardt, 1996, Diekmann et al., 1991). Tyr177 of BCR acts as a docking site for Growth factor Receptor-Bound protein 2 (GRB2) and the ABL1 protein (Wu et al., 1998, Ma et al., 1997).

Therefore, fusion with BCR brings new regulatory domains to the ABL protein and an increased tyrosine kinase activity of ABL (Figure 1.3).

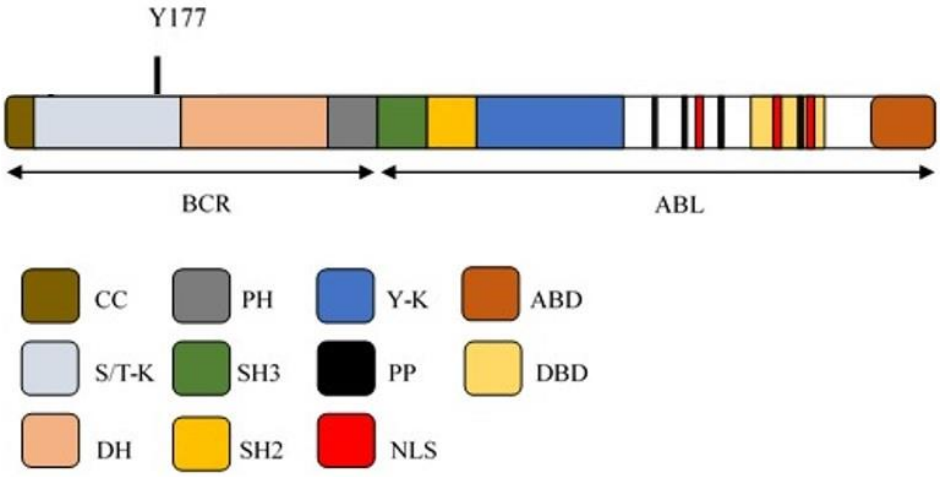

Figure 1.3 Schematic representation of the BCR-ABL kinase This Figure illustrates the structural modularity of BCR-ABL. ABL is composed of a tyrosine kinase (Y-K), a SRC-homology-2 (SH2) domain, a SH3 domain, proline-rich SH3 binding sites (PP), nuclear localization signals (NLS), a DNA-binding domain (DBD) and an actin-binding domain (ABD). BCR is composed of coiled-coil oligomerisation domain (CC), serine/threonine (S/T) kinase, Dbl/CDC24 guanine nucleotide exchange factor homology (DH), and pleckstrin homology (PH) domain, and also has an exposed binding site for other signalling proteins at tyrosine 177 (Y177). The fusion point occurs after the PH domain of BCR (Redrawn from Ren, 2005)).

1.1.4.3 Mechanism of ABL kinase activation

ABL kinases contain two flexible loops which are the ATP-binding loop (p-loop) and the activation loop. These loops are arranged to stabilise the inactive conformation of the protein.

ABL kinases consist of a number of domains: N-terminal myristoylation, SH3, and SH2 kinase. In the inactive conformation of ABL, the N-terminal myristoyl group is positioned on a hydrophobic pocket in the ABL tyrosine kinase domain. This results in a clamped structure that has SH2 and SH3 domains on the back of the protein. This clamping structure hinders the activation loop and obstructs ATP binding. In order to activate ABL kinase, the N-terminus must be unlatched from the hydrophobic pocket to expose the SH2 and SH3 domains. Then, SH2 and SH3 bind to the tyrosine and proline rich regions, respectively, to expose the activation loop. This leads to tyrosine 412 phosphorylation and full activation of the kinase (Figure1.4) (Hantschel et al., 2003, Nagar et al., 2003).

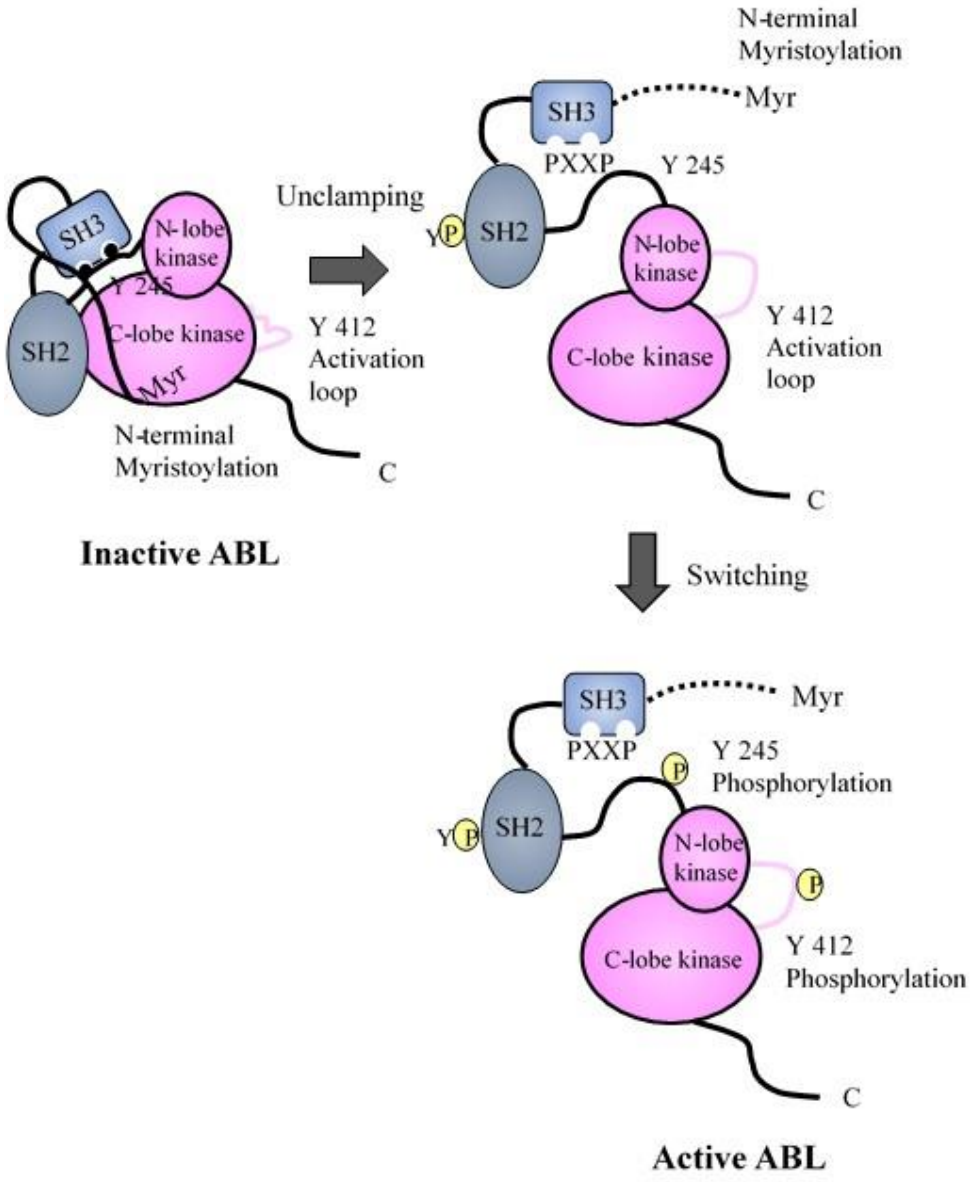

Figure 1.4 Mechanism of ABL kinase activation. The activation loop is exposed in the active conformation of ABL leading to tyrosine phosphorylation and activation of ABL kinase. (Redrawn from (Wong and Witte, 2004)).

1.1.4.4 Signalling pathways downstream of BCR-ABL1 kinase

BCR-ABL activates various downstream signalling pathways. The N-terminal CC domain of BCR-ABL induces dimerization and auto-transphosphorylation (McWhirter et al., 1993). BCR-ABL-mediated leukaemogenesis depends on the autophosphorylation at tyrosine 177 (Tyr177) (Zhang et al., 2001) which normally serves as a docking site for growth factor receptor-bound protein 2 (GRB2) by acting as a high-affinity binding site for the SH2 domain of GRB2 (Pendergast et al., 1993). Then, GRB2 recruits Son of Sevenless (SOS) protein, which is a guanine-nucleotide exchanger of RAS, to trigger RAS activation (Puil et al., 1994, Sattler et al., 2002).

Signalling from RAS activates the mitogen-activated protein kinases (MAPK) to enhance cell proliferation. RAS protein can be activated by a complex of GRB2 and SOS. RAS then activates RAF kinase, which is a serine/threonine kinase that phosphorylates and activates MEK (tyrosine/threonine kinase). Subsequently, MEK phosphorylates and activates MAPK, also known as extracellular signal-regulated kinase (Erk). This RAS-RAF-MEK-ERK pathway is involved in regulation of several transcription factors, such as MYC (Cahill et al., 1996, Marais et al., 1995).

In addition, GRB-2 can form a complex with a scaffold adapter GRB-2 associating binding protein 2 (GAB2) (Sattler et al., 2002). GAB2 then recruits phosphatidylinositol 3-Kinase (PI3K), which activates the phosphoinositide-dependent kinase (PDK-1) and serine/threonine kinase Akt (Skorski et al., 1995, Hemmings and Restuccia, 2012). Akt promotes cell survival, enhances cell proliferation and activates protein translation. These events result in an anti-apoptotic activity within the cells (Franke et al., 1997). In addition, Akt was found to decrease an expression of $p27^{KIP1}$, the CDK2 inhibitor, and thus leads to cell cycle deregulation (Gesbert et al., 2000).

BCR-ABL constitutively activates signal transducer and activation of transcription protein family (STATs) (Ilaria and Van Etten, 1996). STAT5 induces the transcriptional activation of the anti-apoptotic proteins

of the B cell lymphoma 2 (Bcl-2) family, such as Bcl-X_L. As a consequence, this increases the survival of the myeloid progenitors (Figure 1.5) (Quintas-Cardama and Cortes, 2009).

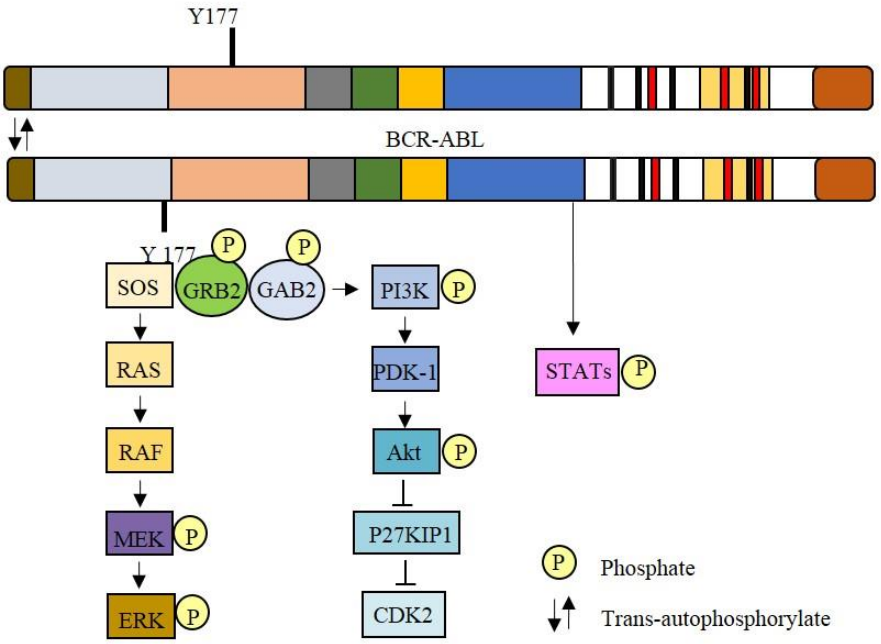

Figure 1.5 Molecular signalling in BCR-ABL–positive myeloid cells. BCR-ABL proteins form a dimer through their CC domains, which results in trans-autophosphorylation to activate many signalling pathways (Redrawn from (Ren, 2005)).

1.1.5 Treatment

Allogenic stem cell transplantation (AlloSCT) is the only way to cure CML. However, this treatment carries a high risk of morbidity and mortality, and also has limitations. It is restricted to patients with good health status and having an appropriate stem cell donor (Druker et al., 2001). As a consequence, drug treatments have been developed as the main therapy for this disease.

Previously, drug therapy for CML disease was limited to non-specific agents such as hydroxylurea, hydroxylcarbamide, busulfan, and

interferon-α (IFN-α) (Wong and Witte, 2004). Of these drugs, IFN-α induced cytogenetic responses in CML patients (Talpaz et al., 1986), improved survival rate of CML patients and induced disease regression, compared to conventional chemotherapy. However, it has relatively high toxicity, and 20 % of CML patients cannot tolerate IFN-α therapy (Salesse and Verfaillie, 2002).

However, the emergence of tyrosine kinase inhibitors (TKIs) totally changed the landscape for the treatment of CML. Imatinib mesylate (Gleevec, STI-571) was developed as a small molecule inhibitor of ABL kinase (Detailed in 1.2.1) and is now used as a frontline therapy for this disease (Vigano et al., 2014)). Despite its clinical success, imatinib-resistant CML has emerged as a result of mutations in the BCR-ABL kinase. Therefore, second generation TKIs were introduced to overcome this resistance, such as nilotinib and dasatinib. These drugs have been approved as first line therapy for newly-diagnosed CML patients by the Food and Drug Administration (FDA). However, these drugs fail to overcome one of the most common mutations, T315I. As a result, third generation TKIs, such as ponatinib have been developed and approved by the FDA. Ponatinib exhibited activity against CML with the T315I (gatekeeper) mutation and therefore is considered as a treatment for imatinib-resistant CML (reviewed in (Deininger et al., 2005, Weisberg et al., 2007, Shamroe and Comeau, 2013)).

Other than ponatinib, non BCR-ABL targeted agents, such as omecetaxine, have been introduced to use in patients who have developed resistance to imatinib. Omacetaxine is a natural alkaloid that inhibits protein synthesis and also induces apoptosis. Omacetaxine induces cytogenetic responses in CML and has recently been approved by the FDA as a treatment option for patients who fail several TKIs (Nazha et al., 2013).

1.1.6 Prognosis

Before the advent of TKIs, CML was considered to be a fatal disease, as the survival time for CML patients was about 3-5 years from diagnosis. Since the use of TKIs, the survival times have improved to over 5 years in most of cases (>89%) (Druker et al., 2006).

1.2 Tyrosine Kinase Inhibitors

In 1845, CML was first described by the pathologists Bennet, Craigie, and Virchow (reviewed in (Wong and Witte, 2004)) but it was only in 1960 that the Ph chromosome was discovered by Nowell and Hangerford (Nowell, 1962) and the molecular basis for the disease was understood. In 1986, Shtivelman and colleagues showed that the Ph chromosome was a BCR-ABL fusion gene via the mapping of both ABL and BCR probes to the mRNA product of the Ph chromosome (Shtivelman et al., 1985, Ben-Neriah et al., 1986, Stam et al., 1987). Therefore, BCR-ABL targeted therapy was investigated for CML treatment.

A decade later, Anafi and colleagues reported that a tyrphostin could inhibit the tyrosine kinase activity of BCR-ABL. Therefore, they suggested that designing specific compounds for the treatment of BCR-ABL associated leukaemia may be possible. However, this compound and its derivatives have not been developed for clinical use because of toxicity issues (Anafi et al., 1993). At around the same time, Lydon and Matterat at Ciba Geigy (now Novartis), initiated a project to identify compounds that inhibit kinase activity. They found that 2-phenylaminopyridine-type tyrosine kinase inhibitor gave a promising result.

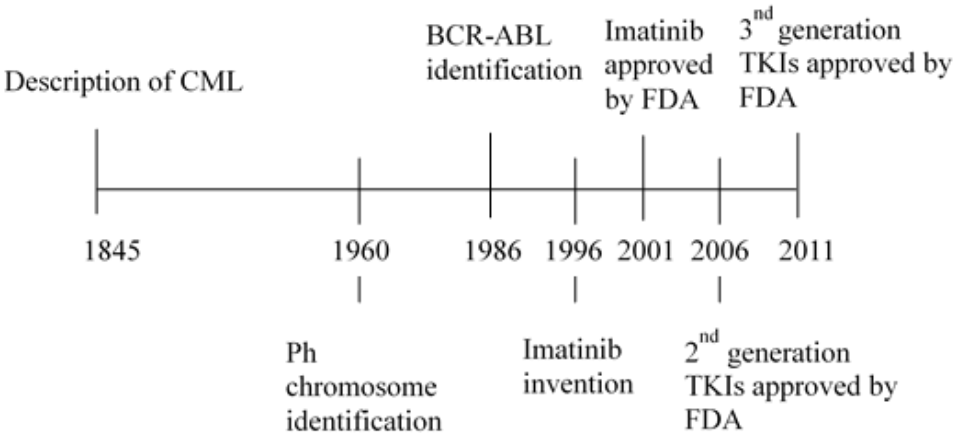

Figure 1.6 Timeline of major discoveries in CML (Redrawn from (Wong and Witte, 2004))

1.2.1 Imatinib

In 1996, imatinib mesylate (Gleevec, STI-571), the specific BCR-ABL inhibitor, was developed and this was a major breakthrough in the treatment of CML (Buchdunger et al., 1996, Druker et al., 1996). Imatinib is a 2-phenylaminopyridine-type tyrosine kinase inhibitor with the addition of a 3'-pyridyl group at the 3' position of the pyrimidine (Fig. 1.7). This pyridyl group enhanced the intracellular inhibitory activity of the drug. In addition, a benzamide group and a flag-methyl group were introduced at the phenyl ring and the diaminophenyl ring, respectively, to improve activity against tyrosine kinases. A N-methylpiperazine group was added to increase oral bioavailability and water solubility (reviewed in (Deininger et al., 2005)) (Figure 1.7).

Imatinib acts by competitive binding to the adenosine triphosphate (ATP) binding site of BCR-ABL protein and stabilising the inactive non-ATP-binding form of BCR-ABL, thus blocking auto-phosphorylation and subsequent signal transduction. This inhibition of BCR-ABL activity results in the anti-proliferative effect of the drug (Schindler et al., 2000). Crystallographic studies showed that imatinib binds to ABL kinase domain through six hydrogen bond interactions and thus prevents ATP from accessing the ATP-binding pocket. The hydrogen bonds involve the following interactions between imatinib and BCR-ABL: the pyridine-N and

M318 backbone-NH; the amide-NH and E286 side chain; the aminopyrimidine and T315 side chain; the carbonyl group and D381 backbone-NH, and the methypiperazine with I360 and H361 (Figure 1.7) (Manley et al., 2002). In addition, there are a number of van der Waals interactions that contribute to this binding. Also, there are interactions between hydrophobic pockets of the protein, and the phenyl ring and the piperazine methyl group of imatinib (Asaki et al., 2006, Eck and Manley, 2009).

Imatinib not only inhibits ABL kinase, but also stem-cell growth factor receptor (c-KIT) and platelet-derived growth factor (PDGFR) (Buchdunger et al., 2000). Imatinib was approved for CML treatment by the FDA in 2001 and it dramatically improves CML patient survival time (Kalaycio, 2001). Imatinib induces complete cytogenetic response in almost 80% of patients diagnosed with CML chronic phase with mild toxicity (Kantarjian et al., 2002).

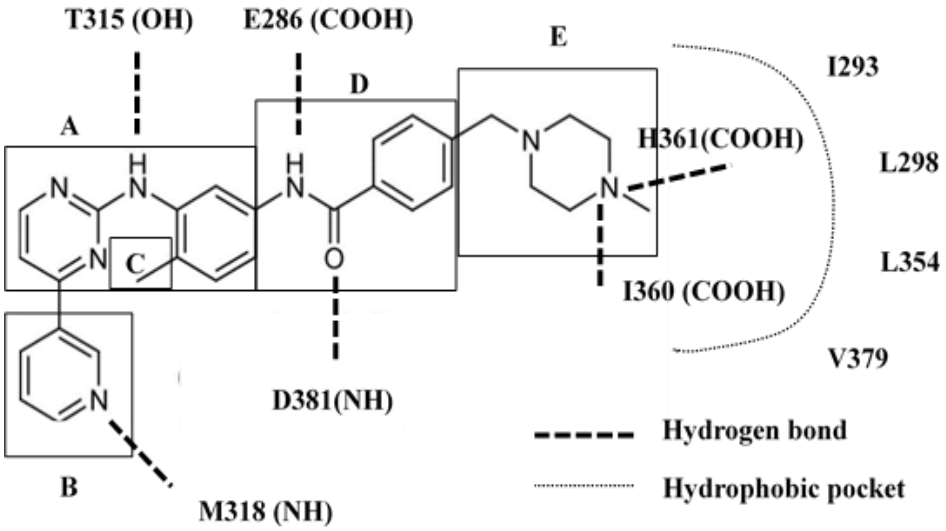

Figure 1.7 Chemical structure and binding site of imatinib melysate to BCR-ABL. The structural features of imatinib are designated as **(A)** 2-phenylaminopyridine backbone **(B)** Pyridyl group **(C)** flag-methyl group **(D)** benzamide group **(E)** N-methylpiperazine group. The key hydrogen bonds and hydrophobic pocket that interact with BCR-ABL are shown in dashed lines, and key residues of BCR-ABL that interact with these imatinib domains are indicated (Redrawn from (Manley et al., 2010)).

Following its initial clinical success, resistance to this drug especially via mutations to BCR-ABL, were observed. Hence, second generation tyrosine kinase inhibitors (TKIs) have been developed such as nilotinib and dasatinib (Deininger et al., 2005).

1.2.2 Nilotinib

A decade after the introduction of imatinib, the second generation TKI, nilotinib (Tasigna, AMN107), was developed by the same company for use as a treatment in BCR-ABL positive CML (Golemovic et al., 2005). Nilotinib is a rationally designed imatinib derivative. A 3-methylimidazole and a trifluoro-methyl group were introduced to enhance its interactions with the Abl kinase domain, compared to imatinib.

Nilotinib also binds to the inactive conformation of ABL kinase. Nilotinib binding with the ABL kinase domain involves four hydrogen bond interactions. These include interactions between: pyridine-N and M318 backbone; the aminopyrimidine and T315 side chain; the amide-NH and E286 side chain; and the carbonyl group and D381 backbone-NH. The 3-methylimidazole and trifluoro-methyl groups stabilise the shape of nilotinib and also bind to the ABL kinase domain with a greater number of van der Waals interactions compared to imatinib (Figure 1.8) (Manley et al., 2010).

Nilotinib is approximately 43-60 fold more potent than imatinib in imatinib-sensitive cell lines, and has a 20-fold greater potency than imatinib in imatinib-resistant cell lines (Golemovic et al., 2005). It displays a similar specificity profile to imatinib as it has a specific activity toward BCR-ABL, c-ABL, and c-KIT targets (Kantarjian et al., 2006, Melo and Chuah, 2008). It has been approved to use as a treatment for both CP and AP and for imatinib-resistant CML patients (Bumbea et al., 2010, Ramirez and DiPersio, 2008).

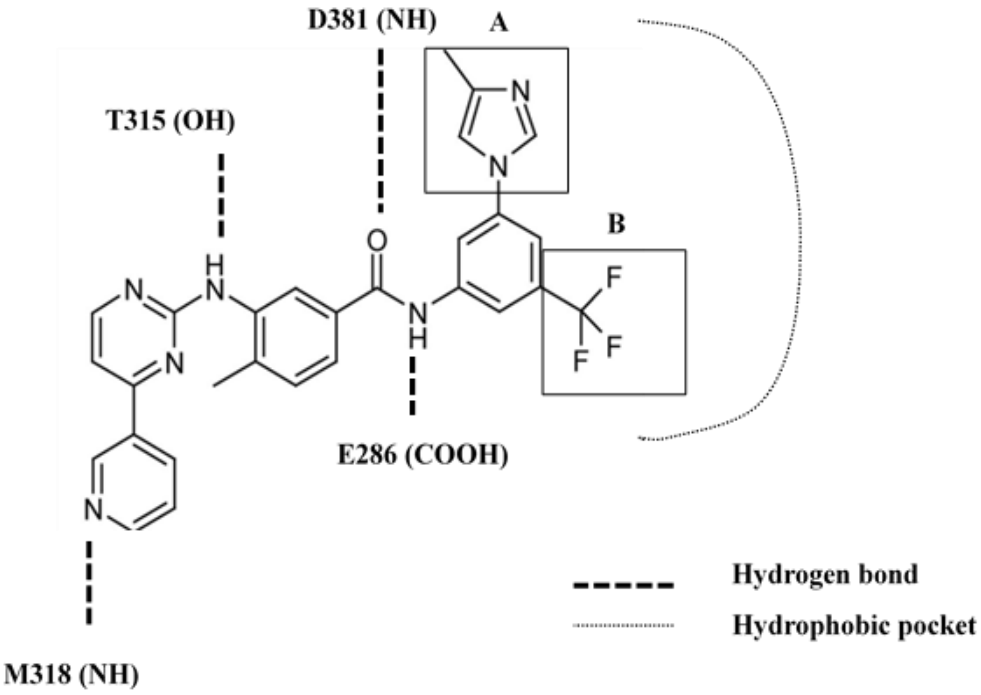

Figure 1.8 Chemical structure and binding site of nilotinib. Nilotinib is a close analogue of imatinib with the addition of **(A)** methylimidazole group **(B)** trifluoro-methyl groups. The key hydrogen bonds and hydrophobic pocket are shown in dashed lines (Redrawn from (Manley et al., 2010)).

1.2.3 Dasatinib

Dasatinib (Sprycel, BMS-354825) is a structurally-distinct TKI that has approximately 350-fold higher potency than imatinib. Dasatinib can bind to both active and inactive forms of BCR-ABL (Tokarski et al., 2006, Das et al., 2006).

Dasatinib is a thiazolylaminopyrimidine analogue. The 2-chloro-6-methyl phenyl ring binds to the hydrophobic pocket of the ABL kinase, and blocks ATP reaching its binding site. Dasatinib requires fewer binding interactions compared to imatinib and nilotinib, leading to a higher potency. Dasatinib binds to ABL kinase via three hydrogen bonds. These hydrogen bonds result from interactions between: the aminothiazole ring and the amide nitrogen of M318; the 2-amino hydrogen and the carbonyl oxygen of M318; the amide nitrogen and the T315 side chain. The other interactions are mainly via van der Waals interactions (Figure 1.9) (Tokarski et al., 2006).

Dasatinib is a multi-kinase inhibitor as it has activity against BCR-ABL, c-Kit, PDGFR, and SRC family kinases, such as mitogen-activated protein kinase and receptor tyrosine kinase (Shah et al., 2004). It is active against many BCR-ABL kinase domain mutations due to it having fewer binding requirements.

It has been approved for use in all phases of CML especially patients who failed previous imatinib treatment (Muller et al., 2009, Hochhaus et al., 2009). This improved potency inhibits most, but not all of the imatinib resistant mutants, especially the T315I mutant (Rix et al., 2007).

Although second generation TKIs exhibit clinical success in most CML cases, emerging mutations, such as T315I still present an obstacle to overcome imatinib resistance. The third generation TKIs, ponatinib and rebastinib, have been developed for this purpose.

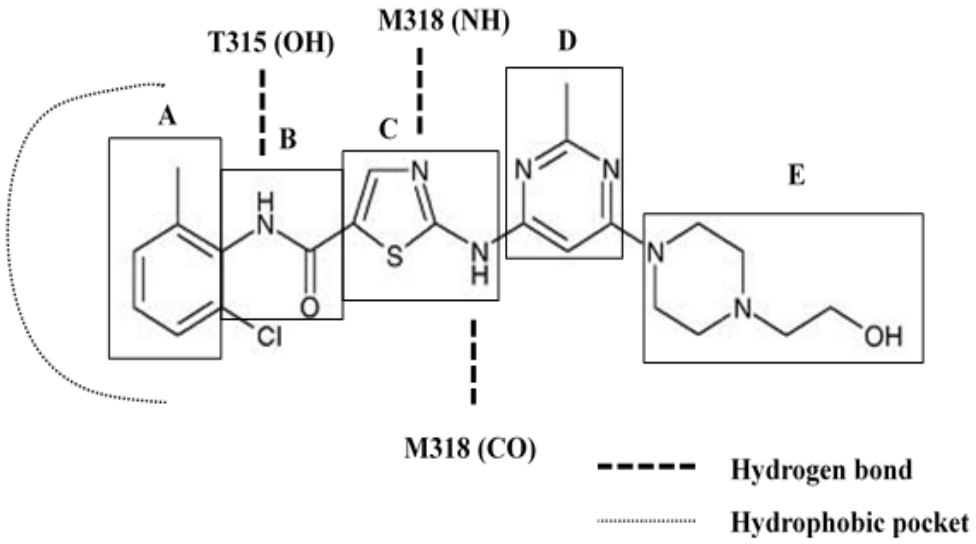

Figure 1.9 Chemical structure and binding site of dasatinib The structure of dasatinib is defined as **(A)** 2-chloro-6-methyl phenyl group, **(B)** amide group, **(C)** aminothiazole group, **(D)** pyrimidine ring, and **(E)** hydroxyethyl-piperazine group. The key hydrogen bonds and hydrophobic pocket are shown in dash lines (Redrawn from (Tokarski et al., 2006)).

1.2.4 Ponatinib

Ponatinib (Iclusig, AP24534) was developed by rational design based on the crystal structure of ABL kinase domains in complex with imatinib. To avoid the interaction with side chain of 315I (i.e. to target the T315I mutation) (detailed in 1.3.1), ponatinib was designed with a linear ethynyl linker in order to bind tightly with ABL kinase and avoid the bulky isoleucine side chain. In addition, ponatinib forms five hydrogen bonds with ABL kinase domain, which are interactions between: the nitrogen atom of the purine group and the backbone of M318; the amide group and backbone of D381 and the E286 side chain; the protonated methylpiperazine and the backbone-carbonyl atoms of I360 and H361. The methyphenyl group binds to the hydrophobic pocket of ABL kinase while the trifluoromethyl group binds to the DFG-out pocket, which is a novel pocket induced by the inactive conformation of ABL kinase domain (Figure 1.10) (Zhou et al., 2011).

Ponatinib also inhibits other kinases such as PDGFR, and c-Kit (Lierman et al., 2012). It is active against a range of TKI resistant CML cells (Cassuto et al., 2012) and has exhibited clinical efficacy in patients with multi-TKIs resistance (Jain et al., 2013). However, some reports have shown that ponatinib resistance has been developed in some cases (Zabriskie et al., 2014).

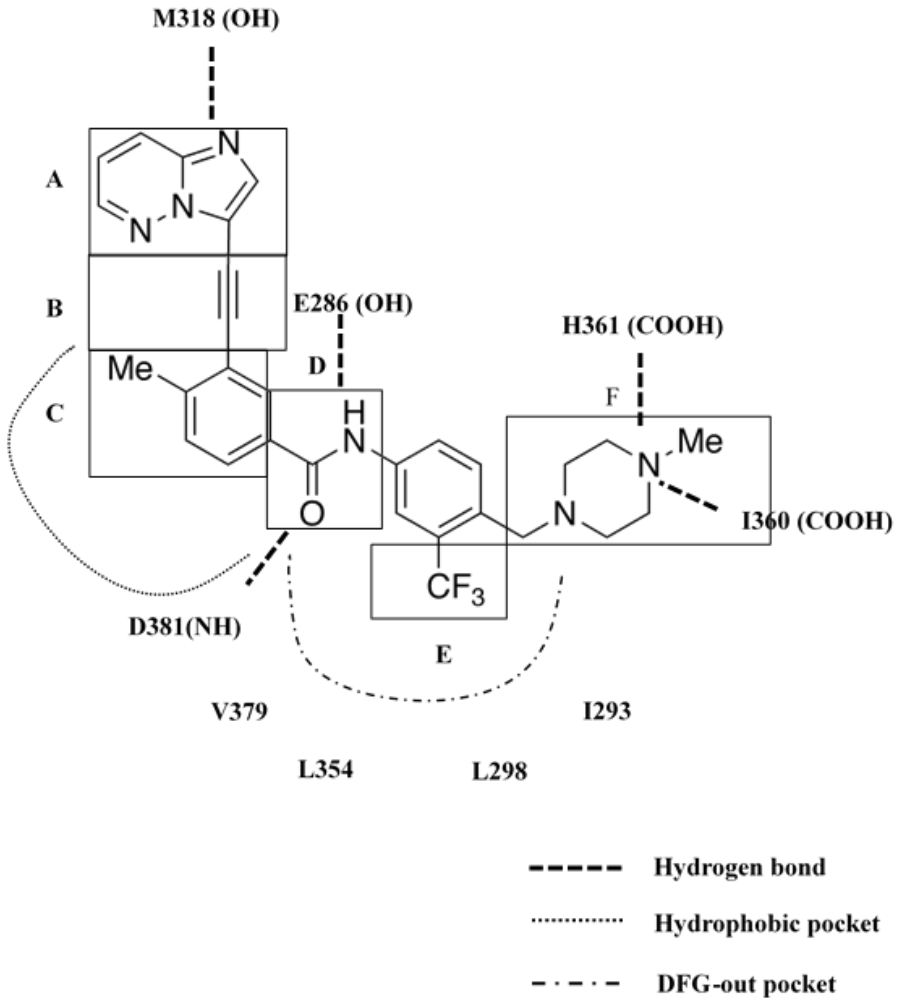

Figure 1.10 Chemical structure and binding site of ponatinib. The structure of ponatinib is defined as **(A)** purine group, **(B)** ethynyl linker, **(C)** methylphenyl group, **(D)** amide group, **(E)** trifluoromethyl group, and **(F)** N-methylpiperazine group. The key hydrogen bonds, hydrophobic pocket, and DFG-out pocket are shown in dashed lines (Redrawn form (Zhou et al., 2011)).

1.3 Tyrosine Kinase Inhibitor Resistance in CML

During the advanced stages of CML, rates of mutations are high, thus resistance to imatinib has emerged by various mechanisms. Therapeutic resistance to imatinib occurs in approximately 10-15 % of patients (Zhang et al., 2009b). This resistance is classified as primary (intrinsic) and secondary (extrinsic) resistance, depending on the time of onset. The primary resistance is defined by a lack of response to imatinib from the onset of the disease, while secondary resistance is defined as a loss of efficacy of imatinib after an initial response (Mauro, 2006). In this section, some mechanisms leading to imatinib resistance are reviewed.

1.3.1 BCR-ABL Oncogene Amplification

BCR-ABL oncogene amplification was the first mechanism of imatinib resistance to be identified. In 2000, Mahon and colleagues found an amplification of Abl sequences in BCR-ABL-positive cells, leading to imatinib resistance. This amplification resulted in an elevated level of BCR-ABL protein and thus decreased the effectiveness of imatinib at clinically-used doses (Mahon et al., 2000, le Coutre et al., 2000).

1.3.2 BCR-ABL1 kinase domain mutations

40-90 % of imatinib-resistance patients develop BCR-ABL1 kinase domain mutations (Corbin et al., 2003). These mutations cause imatinib resistance by either impairing imatinib binding with BCR-ABL kinase or indirect modulation of kinase function. These mechanisms are usually associated with secondary imatinib resistance (Zhang et al., 2009b).

35-70 % of patients displaying imatinib resistance have developed point mutations (Jabbour et al., 2006). More than 100 different point mutations of BCR-ABL kinase domain have been reported so far (Rix et al., 2007). The regions that are important for imatinib binding are often the regions in which acquired mutations are reported. These regions are the P-loop, tyrosine 315 (T315), methionine 351 (M351), glutamate 355 (E355), and the activation loop. These mutations all contribute to the activated

conformation of imatinib-insensitive ABL kinase. Soverini and colleagues reported that most of the mutations associated with imatinib resistance are related with amino acid substitution at one of seven residues (Soverini et al., 2011). These residues are M244V, G250E, Y253F/H, and E255K (in the P-loop); T315I (at the TKI contact site); M351T and F359V at the catalytic domain However, the most important of these mutations, are the T315I and P-loop mutations.

1.3.2.1 T315I mutation

T315 is a non-conserved residue that is located in the binding pocket of ABL kinase and imatinib. The T315I mutant is a frequently observed mutation in imatinib-resistant CML patients (Apperley, 2007). It results from a single amino acid substitution at position 944 of the ABL kinase leading to a threonine to isoleucine substitution.

In general, TKIs form a key hydrogen bond interaction with Tyrosine 315 in the BCR-ABL protein. Therefore, this mutation will impair this H-bond interaction and hence TKIs binding (Gorre et al., 2001). This mutation is associated with poor prognosis and various studies have reported that it promotes disease progression (Azam et al., 2008, Nicolini et al., 2013). In addition to imatinib, all second generation TKIs, such as dasatinib and nilotinib cannot inhibit this T315I mutant, as they require the same critical hydrogen bond with T315 of ABL kinase for binding.

1.3.2.2 P-loop mutations

The P-loop is a highly conserved region that is responsible for phosphate binding. P-loop mutations induce inactive ABL to transform into active ABL with intrinsic kinase activity. These P-loop mutations are frequently associated with the progression from CP, and from AP to BP (reviewed in (O'Hare et al., 2007, Comert et al., 2013)).

1.3.2.3 Other mutations

The BCR-ABL is composed of many domains, such as SH2, SH3, and DNA-binding domains and several *in vitro* studies have shown that mutations of these sites can also confer imatinib resistance. These domains exhibit auto-inhibitory effects on ABL kinase; therefore their mutation can result in the auto-activation of ABL kinase. In addition, some mutations are located at substrate binding sites, such as the activation loop (Roche-Lestienne et al., 2002, Soverini et al., 2006).

These mutations cause imatinib-resistance, but they are not as common as the T315I and the P-loop mutations.

1.3.3 BCR-ABL independent mechanisms

BCR-ABL independent mechanisms can also result in TKIs resistance and development of multidrug resistance. These mechanisms include decreased TKIs influx, increased TKIs efflux, and other events that result in loss of effectiveness of drugs.

1.3.3.1 Modulation influx transport

Impairment of imatinib uptake can result in failure to inhibit CML cell proliferation because an effective inhibitory concentration of imatinib cannot be achieved within the cell. The organic cation transporter-1 (Oct-1) has been reported to be responsible for imatinib transport into BCR-ABL positive cells (White et al., 2006). There is evidence that alteration in the expression and function of Oct-1 leads to imatinib-resistance in CML (White et al., 2012, Engler et al., 2010).

1.3.3.2 Modulation efflux transport

Impairment of imatinib efflux is another way to prevent imatinib from attaining its inhibitory cellular concentration. ATP binding cassette (ABC) transporter family members include Polyglycoprotein multidrug resistance 1 (ABCB1), multidrug resistance-associated proteins 1 (ABCC1), and breast cancer resistance protein (ABCG2) and are all drug efflux transmembrane proteins that regulate drug transport. There is a report

showing that the overexpression of ABCB1 and ABCG2 contributes to imatinib-resistance in CML (Dohse et al., 2010).

1.3.3.3 Modulation of survival mechanisms

The increased resistance to apoptosis of CML cells is regulated by several survival mechanisms, such as a deregulation of Bcl-2 family proteins and an increased expression of inhibitors of apoptosis proteins (IAPs) (reviewed in (Rumjanek et al., 2013)).

1.4 Apoptosis

The term of apoptosis is used to describe the ordered sequence of events that a cell undergoes in response to certain death-inducing stimuli (Kerr, 1999). Apoptosis is associated with physical and biochemical changes in the cytoplasm, plasma membrane and nucleus of cells. The apoptotic cell morphology is defined by cell rounding, nuclear fragmentation, blebbing of the plasma membrane, loss of mitochondrial membrane potential and chromatin fragmentation (Ziegler and Groscurth, 2004). The biochemical changes that occur in apoptosis are divided into three main categories: (1) membrane changes and recognition of apoptotic cells by phagocytic cells; (2) breakdown of DNA and cellular proteins and (3) caspase activation.

In early apoptotic cells, phosphatidylserine, which is normally located on the inner plasma membrane of the cell, becomes externally exposed. This external expression is recognised by phagocytic cells and results in the phagocytosis of the apoptotic cell, normally without activating an inflammatory response. Other features of apoptosis include DNA breakdown via endonuclease activation and caspase activation. This latter event results from the activation of the group of cysteine proteases, which can cleave a number of cellular proteins (reviewed in (Wong, 2011)).

Apoptosis is classified into two major pathways; death-receptor-mediated (extrinsic) and mitochondrial-mediated (intrinsic) pathways.

1.4.1 Receptor-Mediated Apoptosis (Extrinsic pathway)

The extrinsic pathway of apoptosis is one of the major mechanisms that results in cell death. It is activated when extracellular ligands bind to plasma membrane receptors to ultimately trigger apoptosis. The typical death receptors are Fas (Apo-1 or CD95) and Tumour Necrosis Factor Receptor (TNF-R), and their ligands are Fas ligand (FasL) and TNF, respectively. These receptors recruit adapter proteins such as Fas-associated death domain (FADD) and TNF receptor associated death domain (TRADD), as well as cysteine protease (caspase 8) via their intracellular death domains.

These receptor-ligand-adaptor protein complexes are termed death-inducing signalling complexes (DISC), which then activate pro-caspase 8 to become an initiator caspase. This initiator caspase then activates the executioner caspases to degrade cellular targets (Schneider and Tschopp, 2000).

1.4.2 Mitochondrial-Mediated Apoptosis (Intrinsic pathway)

The intrinsic pathway is activated within cells by various internal stimuli or growth factor/cytokine depletion. These internal stimuli include genetic damage, high concentrations of cytosolic calcium, hypoxia, and oxidative stress. This pathway is often activated when the cell no longer receives growth factors/cytokines or when there is disruption in the expression/localisation/activity of members of the B cell lymphoma 2 (Bcl-2) family of proteins (see below). It results in disruption of the potential of the inner mitochondrial membrane and the release of anti-apoptotic molecules, such as cytochrome *c* into the cytosol to bind with apoptotic protease activation factor-1 (Apaf-1). This binding, together with ATP, triggers apoptosome formation, which contains seven molecules of Apaf-1, seven cytochrome *c* molecules, and seven Procaspase-9 molecules. This results in activation of the initiator apoptosome-bound, procaspase-9. This then activates downstream effector caspases, particularly caspase-3, to cleave cellular targets (Lawen, 2003, Danial and Korsmeyer, 2004). Other apoptotic factors that are released from the space between the outer and

inner mitochondrial membranes into the cytoplasm include mitochondria-derived activator of caspases (Smac) and direct IAP binding protein with low pI (DIABLO). These proteins can also promote caspase activation via binding to inhibitor of apoptosis proteins (IAP). This contributes to inhibition of the interactions between IAPs and caspase-3 and/or caspase-9 (Kroemer et al., 2007, LaCasse et al., 2008).

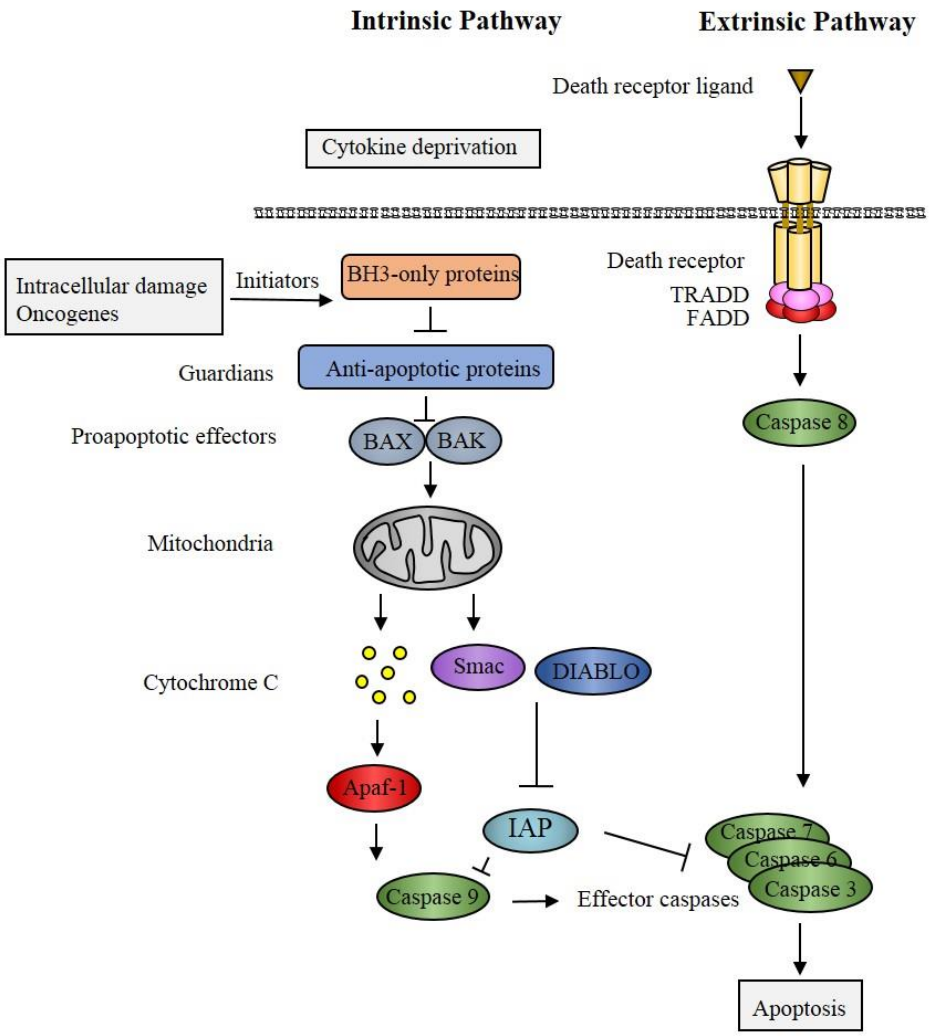

Figure 1.11 The extrinsic and intrinsic pathways of apoptosis The extrinsic pathway is activated by death receptor ligands binding to death receptors leading to activated caspase 8 by the receptor-associated death domains. Then, effector caspases are activated by caspase 8. The intrinsic pathway is regulated by Bcl- 2 family proteins. BH-3 family members (initiators) are activated and then inhibit pro-survival Bcl-2 family (guardians), thus enabling activation of BAX and BAK (pro-apoptotic effectors), which form pores in the mitochondrial membrane to release cytochrome C. Cytochrome C then promotes caspase 9 activation through APAF-1 to activate effector caspases (Redrawn from (Czabotar et al., 2014)).

1.5 Apoptosis and carcinogenesis

Delayed or resistance to apoptosis can lead to cancer development. In general, the mechanisms that alter apoptosis in cancer cells can be divided into three main categories: (1) disruption of pro-apoptotic and anti-apoptotic proteins; (2) decreased caspase activity and (3) impairment of death receptor signalling.

1.5.1 Disruption of pro-apoptotic and anti-apoptotic proteins

Anti-apoptotic members of the Bcl-2 family are often located at the outer membrane of mitochondria, where they function to preserve to mitochondrial membrane permeability (Martinou and Youle, 2011).

The Bcl-2 protein family is divided into three subgroups based on their pro- or anti-apoptotic function and the number of Bcl-2 Homology (BH) domains that they possess. Bcl-2 family proteins have motifs of sequence homology known as BH domains. Some pro-survival family members have four BH domains (except Mcl-1 that has only three BH domains) and adopt similar globular structures. However, BH3-only family members express only the BH3 domain (Kvansakul et al., 2008, Hinds et al., 2007) (Figure 1.12).

Changes in the function or localization of these proteins results in a deregulation of apoptosis by an imbalance in the interactions of pro-apoptotic and anti-apoptotic proteins. This can result from over-expression of an anti-apoptotic protein or under-expression of a pro-apoptotic protein, or a combination of both. There are various reports that show that deregulation of expression of Bcl-2 family proteins is involved in many types of cancer, such as neuroblastoma, glioblastoma, breast cancer, and CML (reviewed in (Frenzel et al., 2009).

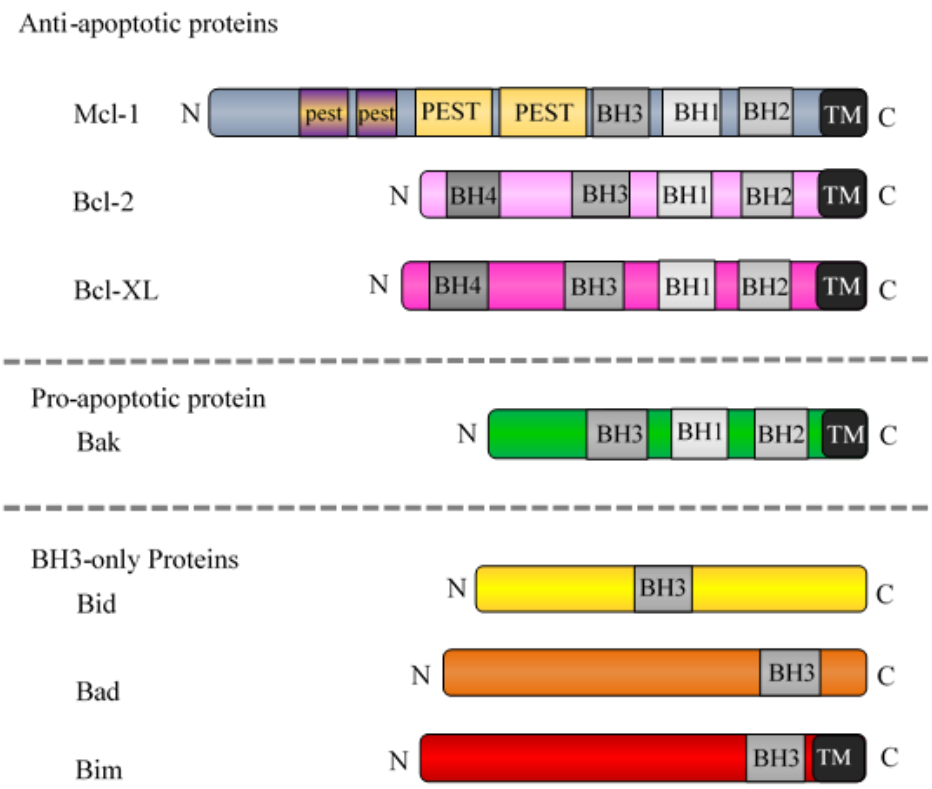

Figure 1.12 Sequence homology and structure of Bcl-2 family proteins Some anti-apoptotic proteins possess four BH domains (BH1-BH4) while the BH3-only proteins possess only the BH3 amphipathic helix, which allows them to interact with other Bcl-2 family member proteins (Redrawn from (Czabotar et al., 2014)).

1.5.1.1 Pro-survival or Anti-apoptotic Bcl-2 Family Members

This subgroup includes Bcl-2, Bcl-X_L, Bcl-w, Bfl-1/A1, and myeloid cell leukaemia sequence 1 (Mcl-1) (Figure 1.12). They all possess anti-apoptotic functions by preventing mitochondrial outer membrane permeabilisation (MOMP). Structural studies have revealed that BH domains (BH1, BH2, and BH3) of these anti-apoptotic proteins possesses a hydrophobic groove on their surface and thus act as a binding site for the α-helical BH3 domain of pro-apoptotic proteins, in order to neutralize their pro-apoptotic action (Petros et al., 2004).

They protect mitochondrial membrane integrity and inhibit apoptosis via inhibiting other pro-apoptotic proteins, such as Bak and Bax, which may otherwise form pores in the mitochondrial membrane via oligomerisation (Khemtemourian et al., 2006). Anti-apoptotic family members also therefore prevent activation of various initiator caspases and prevent Apaf-1 and caspase 9 activation (reviewed in (Youle and Strasser, 2008)).

Mcl-1 protein has unique characteristics among the anti-apoptotic subgroup of Bcl-2 family, due to its short half-life and ability to undergo post-translational modifications in its unique N-terminal domain. This domain contains many sites that are subject to reversible modifications, such as ubiquitination and phosphorylation (Figure 1.13) (Akgul, 2009). In addition, it has a relatively large size (350 amino acids and residues) compared to other members in Bcl-2 family, such as Bcl-2 (239 residues) and Bcl-X_L (233 residues) (Day et al., 2005). Mcl-1 protein levels are dynamically regulated in cells (via changes in its rate of synthesis and turnover) and it is an important regulator of cell survival, particularly in cancer cells.

Over-expression of Mcl-1 is associated with a number of malignancies that include several types of tumours, including CML (Aichberger et al., 2005).

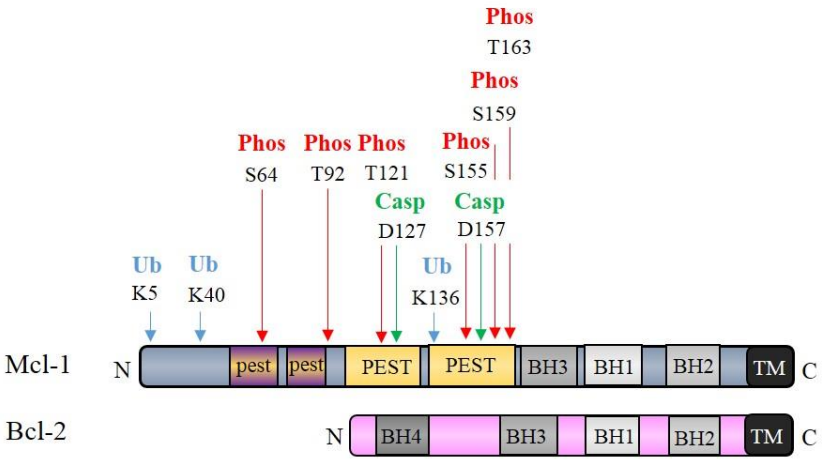

Figure 1.13 A schematic representation of the functional domains and sites of post-translational modification of Mcl-1 protein. The transmembrane (TM), the Bcl-2 homology domains (BH1-3), two weak (lower case) and two strong (upper case) PEST sequences are highlighted along with post-translational sites, including ubiquitination (Ub), caspase cleavage (Casp), and phosphorylation (Phos). A schematic representation of Bcl-2 protein is also shown below to compare the relative size and domain organization (Redrawn from (Thomas et al., 2010)).

1.5.1.2 Pro-apoptotic Family Members

This subfamily includes Bak, Bax, and Bok. Their protein oligomers can insert into the mitochondrial membrane which results in pore formation and the release of the apoptotic factor, cytochrome c, leading to activation of caspases. These activated caspases then cleave specific cellular proteins leading to cell death (Petros et al., 2004).

1.5.1.3 BH-3-only Family Members

The proteins in this subgroup share similarity only in the BH3 domain, and comprise proteins, such as BH3-interacting domain death agonist (Bid), Bim, Bad, Bik, Bmf, Hrk, Noxa, Diva, and Puma. In response to intracellular signals, BH3-only proteins initiate apoptosis by either inhibiting anti-apoptotic proteins or activating pro-apoptotic proteins (Chen et al., 2005, Willis et al., 2005, Willis et al., 2007, Letai et al., 2002, Kuwana et al., 2005). BH3-only proteins bind to the hydrophobic groove of anti-apoptotic proteins and pro-apoptotic proteins in order to neutralise (Liu et al., 2003, Petros et al., 2004).

Bid has a unique activation mechanism among this subgroup because it interconnects the death receptor mediated pathway and the mitochondrial mediated pathway (Kaufmann et al., 2007). The structure of Bid is different from other family members because it has a key BH3 residue that is normally inactivated (Chou et al., 1999, McDonnell et al., 1999). After death receptor simulation, Bid is then cleaved by caspase 8 into tBid, which then induces apoptosis by enhancing oligomerisation of pro-apoptotic proteins (Wei et al., 2001, Eskes et al., 2000).

1.5.1.4 Bcl-2 family in Chronic Myeloid Leukaemia

Many studies have shown that BCR-ABL positive cells are relatively resistant to apoptosis. In part, this may be explained by the fact that BCR-ABL activates many signalling pathways, some of which regulate the expression of Bcl-2 protein members.

There many reports that show that altered expression of anti-apoptotic proteins is involved in CML pathology. For example, the level of expression of the anti-apoptotic protein, Bcl-2, was downregulated after treatment with imatinib (Jacquel et al., 2003), suggesting that this protein is involved in CML pathogenesis. Moreover, levels of expression of another anti-apoptotic protein, Bcl-X_L were increased by activation of the PIK-3/Akt pathway through BCR-ABL (Tzifi et al., 2012). Another study showed that Mcl-1 expression was higher in BCR-ABL expressing cells, when compared to normal cells and imatinib decreased the level of Mcl-1 expression in CML cell lines (Aichberger et al., 2005).

In addition to the effects of BCR-ABL on expression of anti-apoptotic protein, pro-apoptotic proteins are also affected. BCR-ABL expression prevents translocation of the pro-apoptotic proteins, Bad and Bax, and binding with the mitochondrial membrane. This loss of apoptosis control can lead to more unregulated cell growth and development of TKI resistance (Keeshan et al., 2002).

1.5.2 Decreased caspase activity

The caspases that play a role in apoptosis can be classified into initiator caspases and effector caspases. The initiator caspases (caspase-2, -8,-9, and -10) are primarily responsible for initiation of apoptosis pathway, while the effector caspases (caspase-3, -6, and -7) are responsible for the cleavage of cellular targets (Fink and Cookson, 2005).

Down-regulation of caspase activity has been reported in many types of cancer. For example, the down-regulation of caspase-9 has been frequently found in colorectal cancer patients (Shen et al., 2010). In addition, there is a report showing that deficiency of caspase-3 leads to breast cancer cell survival and resistance to chemotherapeutic agents (Devarajan et al., 2002).

1.5.3 Impairment of death receptor signalling

Death receptors and their ligands play a central role in the extrinsic pathway of apoptosis. These receptors possess a death domain that can bind to various effector molecules after the cell receives a death signal. This results in the activation of a signalling cascade leading to caspase activation. A number of abnormalities of death receptors have been reported in leukaemia, including the down-regulation of death receptor expression and the impairment of death receptor function. For example, the down-regulation of the Fas receptor was found in treatment-resistant leukaemia (Friesen et al., 1997).

1.6 Novel Agents in CML Therapy

A number of new approaches and agents have been developed as alternative treatments for imatinib-resistant CML. These include modifications/derivatives of conventional therapeutic agents (new generations of TKIs) and other protein kinase inhibitors which target activated signal transduction pathways in CML.

Inhibiting the downstream signalling pathways of BCR-ABL activity is an important strategy to improve imatinib-resistant CML therapy. There are several studies showing that the combination of the conventional TKIs and inhibitors that down-regulate BCR-ABL signalling pathways leads to an additive or even synergistic effect in imatinib-resistant CML models. For example, the combination of imatinib and OSU03012, a PDK-1 inhibitor, has been shown to induce synergistic effects in imatinib-resistant cell lines (Tseng et al., 2005). As a result of some of these additive and synergistic drugs effect in CML cell models, some of these inhibitors have now entered clinical trials for CML patients (reviewed in (Deininger et al., 2005)). In addition, other inhibitors that target different pathways are also reported for use in CML, including aurora kinase inhibitors and proteasome inhibitors, as described in the following sections.

1.6.1 PI3K inhibitors

Phosphatidylinositol 3-kinases (PI3K) are a family of lipid kinases that mediate intracellular signalling from cell surface receptors. One such inhibitor, CAL-101 (GS11-01), is a PI3K inhibitor that has shown preclinical activity in CML cell lines. CAL-101 enhanced apoptosis in CML cell lines when used in combination with nilotinib (Airiau et al., 2013). Moreover, another study showed that the PI3K inhibitor, wortmannin (KY12420), enhanced the effect of imatinib in induction of apoptosis in imatinib-resistant CML cell lines (Klejman et al., 2002).

1.6.2 Cyclin Dependent Kinase inhibitors

A recent report supported the idea that CML is associated with altered CDK activity, particularly CDK2. CDKs are a family of serine/threonine kinases that are involved in cell cycle progression (Diallo and Prigent, 2011). A number of oncogenes in CML, such as ras, target CDKs leading to their inappropriate activation. Thus, targeting CDKs is a promising future avenue for CML treatment (Cortez et al., 1997) (more details are described in section 1.7).

1.6.3 Aurora kinase inhibitor

Aurora kinases comprise a family serine/threonine kinases that are essential for cell proliferation. They play a role in cell division by controlling chromatid segregation while their over-expression has been found in various types of cancers, such as leukaemia and colon cancer (Fu et al., 2007). There are three major types of aurora kinase: aurora kinases A, B, and C. Aurora kinase A is involved in mitotic spindle formation and centrosome maturation, while aurora kinase B is involved in binding of the mitotic spindle to the centromere (Giet and Prigent, 1999). However, the function of aurora C is still unclear (Katayama et al., 2003).

The pan inhibitor of aurora kinases, danusertib, has entered phase II clinical trials in imatinib-resistant CML patients, as it has been found to inhibit the T315I mutant of BCR-ABL (Gontarewicz et al., 2008a, Gontarewicz et al., 2008b).

1.6.4 Proteasome inhibitors

The ubiquitin-proteasome pathway provides a major intracellular mechanism to degrade cellular components and is thus critical for cell survival and function. Proteasome inhibitors suppress proteasomal degradation of several cell cycle regulator proteins, as well as Bcl-2 family proteins (reviewed in (Crawford et al., 2011)).

The proteasome inhibitor, bortezomib, inhibits cell proliferation and induces apoptosis in imatinib-resistant CML cells (Gatto et al., 2003, Soligo et al., 2001). Moreover, the combination of proteasome inhibitor with imatinib and other agents such as CDK inhibitors, showed synergistic effects on growth inhibition in imatinib-resistant CML cell lines *in vitro* (Yu et al., 2003, Dai et al., 2004).

1.7 Cyclin-Dependent Kinases and Cell Cycle Regulation

Cyclin-dependent kinases (CDKs) play important roles in the sequence of events that control progression through the cell cycle. Deregulation of CDK activity results in various proliferative disorders, especially cancer.

1.7.1 Control of the Cell Cycle

Cell division is defined by DNA replication and segregation of replicated chromosomes into two separated cells. Cell division consists of 2 stages: (1) mitosis (M phase) and (2) the interphase between mitosis. The M phase consists of prophase, metaphase, anaphase, and telophase, while interphase can be divided into G1, S, and G2 phases (Norbury and Nurse, 1992). DNA replication occurs in the S phase which is preceded by G1 gap (a gap for cell preparation) and is followed by G2 Gap (a gap for cell preparation for mitosis). In addition, there is a G0 Gap which is a cell resting stage. Therefore, a cell in G1 phase can enter G0 phase and stop growing and proliferating (Figure 1.14).

Transition between phases of cell cycle is regulated by different cellular proteins. CDKs are the key regulatory proteins which are activated by cyclins at specific points of the cell cycle. Activated CDKs then phosphorylate various proteins (Morgan, 1995).

1.7.2 CDK Diversity and Function

Several CDKs have been identified in mammalian cells. Each CDK contains a catalytic core, which remains inactivated until it forms a complex with its cyclin partner. This generates a conformational change in the CDK to expose its ATP-binding site (Fisher and Morgan, 1994). In addition to cyclin binding, CDKs are required to be phosphorylated at conserved tyrosine and threonine residues by cdk-activating kinase (CAK; cyclin H and CDK7) which results in conformation changes for enhanced cyclin binding (Jeffrey et al., 1995). CDKs functions in cell cycle regulation are described in Table 1.2.

Table 1.2 Mammalian CDKs and Cyclins (reviewed in (Vermeulen et al., 2003, Fischer and Lane, 2000))

CDK	Associated cyclins	Function
CDK1 and CDK2	A	G2-M transition and S phase progression
CDK1	B1 and B2	G2 phase exit and mitosis
CDK2	E	G1-S phase transition
CDK4 and CDK6	D1, D2, and D3	G0-S phase transition
CDK5	G1 and G2	DNA damage response
CDK7	H	CDK activation (CAK), transcriptional regulation, and DNA repair
CDK9	T1 and T2	Transcriptional regulation

CDKs can be divided into two groups based on their roles in cell cycle progression and transcriptional regulation (Meyerson et al., 1992). The first group that is involved in cell cycle progression includes CDK1, 2, 4, and 6. CDK4 and 6 are associated with cyclin D to facilitate G1 entry (Sherr, 1994), while the CDK2/cyclin E complex facilitates G1 to S phase transition (Ohtsubo et al., 1995). The CDK2 and cyclin A complex is required for S phase progression (Girard et al., 1991). CDK1 can form a complex with either cyclin A or cyclin B in order to facilitate G2 to M phase transition and M phase progression, respectively (King et al., 1994) (Figure 1.16). This activation is regulated by phosphorylation via CAK (Harper and Elledge, 1998).

The other group of CDKs that is involved in transcriptional regulation consists of CDK7/cyclin H and CDK9/cyclin T. They promote initiation and elongation of RNA transcripts through phosphorylating RNA polymerase II (Palancade and Bensaude, 2003).

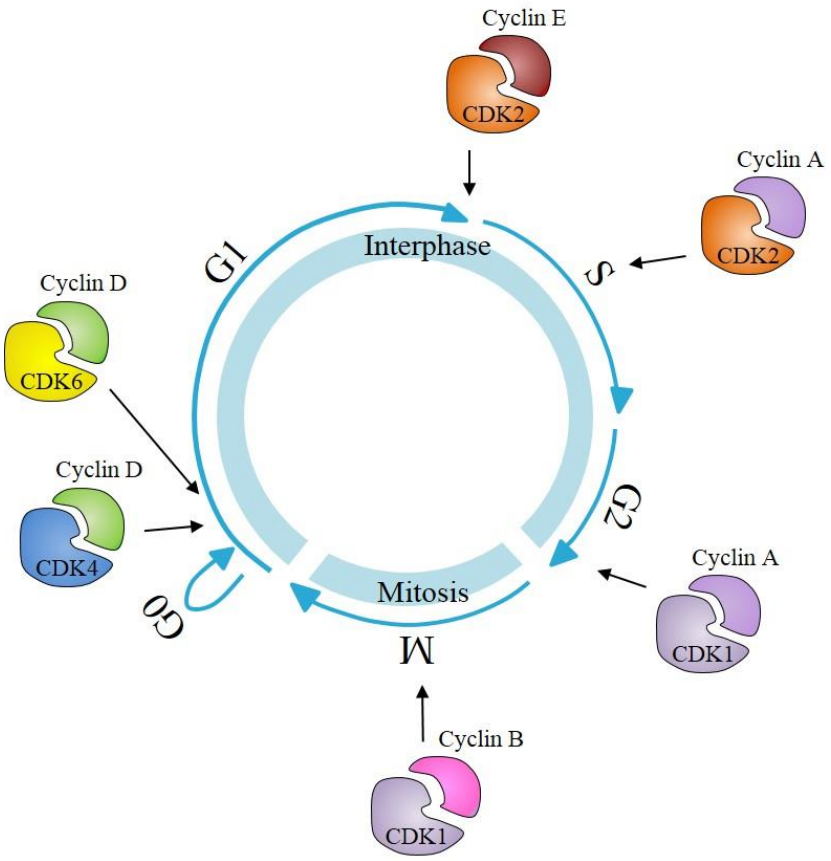

Figure 1.14 The stages of cell cycle. The sites of cell cycle progression regulated by CDK and their associated cyclins are indicated (Redrawn from (Vermeulen et al., 2003)).

1.7.3 CDK Inhibitors in Anti-cancer Drug Development

In view of the fact that CDKs and their regulators are altered in many types of cancer, the development of chemical CDK inhibitors as potential anti-cancer agents has been a major focus for drug discovery. Two different strategies have been utilised; inhibiting CDK activity (direct strategy) and targeting CDK regulators (indirect strategy). However, the direct strategy has had far more success to date than the indirect strategies. More than 50 inhibitors that can inhibit CDK activity have been discovered so far (reviewed in (Fischer and Lane, 2000)), and some of these are in clinical trials. These CDK inhibitors exhibit ATP antagonist activity by binding to the ATP- binding site of the CDKs. CDK inhibitors that have been developed so far are summarized below.

1.7.3.1 Purine Analogues

The natural phytohormone, dimethylaminopurine, was first identified as a CDK inhibitor that inhibits CDK1/cyclin B complex activity (Neant and Guerrier, 1988). This finding led to the development of the synthetic purine analogue, olomoucine, which is a highly specific CDK inhibitor (Vesely et al., 1994). Olomoucine has been found to inhibit cell proliferation and induce apoptosis in various types of cancer by inhibiting both CDK1/cyclin B and CDK2/cyclin E/A complexes (Abraham et al., 1995).

From the success of olomoucine in CDK inhibition, another synthetic purine, roscovitine, has been developed. Roscovitine generates more potent inhibitory effects with CDK1/cyclin B complex and CDK2/cyclin E/A complex compared to olomoucine. It has a strong anti-proliferative effect, and now has entered clinical trials for solid tumour treatment (Meijer et al., 1997, Clough, 2002, Cicenas et al., 2014). Another purine-based molecule that exhibits CDK inhibitory effects is purvalanol A which is a trisubstituted purine that has been found to inhibit CDK2 activity (Gray et al., 1998) (for more detail see section 1.8).

1.7.3.2 Butyrolactone

Butyrolactone was isolated from the fungus, *Aspergillus* strain F-25799. It is a CDK inhibitor which exhibits inhibitory effects on CDK1, CDK2 and CDK4 (Kitagawa et al., 1993). Butyrolactone I inhibits cell cycle proliferation in lung-, colon-, and pancreatic-cancer cell lines (Kitagawa et al., 1994, Yamamoto et al., 1998).

1.7.3.3 Flavonoids

Flavonoids have been found to inhibit various protein kinases, especially protein kinase C. The most important flavonoid that inhibits CDK activity is flavopiridol, which is a synthetic analogue of a natural plant alkaloid. It has been found to inhibit CDK1, CDK2 and CDK4 activities (Carlson et al., 1996). Before entering clinical trials, flavopiridal showed an inhibitory effect on many cell lines, such as breast and lung cancer cell lines (Kaur et al., 1992). It has entered phase I and phase II clinical trials and has been found to decrease cancer cell proliferation in solid tumours, pancreatic cancer, and leukaemias (Carvajal et al., 2009, Blum et al., 2010, Lin et al., 2009).

1.7.3.4 Paullones

Paullones were discovered during an *in vitro* anti-proliferative drug screening programme by National Cancer Institute (NCI). It has been found to inhibit CDK1 and CDK2. Among this group of compounds, kenpaullone was identified as an inhibitor of CDK1/cyclin B, CDK2/cyclin A and CDK2/cyclin E complexes by arresting cell cycle progression at the G1/S boundaries in breast cancers (Zaharevitz et al., 1999).

1.7.3.5 Indolinones

Indirubin was originally isolated from a Chinese herbal mixture and has been used to treat CML. Recently, indirubin and its analogues have been found to exhibit inhibitory effects to CDK1, CDK2, CDK4, and CDK5, and to induce cell cycle arrest at the G2/M phase boundaries (Hoessel et al., 1999). 5'-OH-5-nitro-idirubin oxime (AGM130) is a

synthetic idirubin derivative that efficiently decreases the viability of imatinib-resistant CML cell lines (Kim et al., 2013).

1.7.3.6 Non-specific CDK inhibitors

This group of compounds is characterized by non-specific effects that inhibit protein kinase activities. Thus, not only CDK activities are inhibited, but also other protein kinases such as protein kinase C. This group includes suramin, quinazolines, aminothiazole, and staurosporine. Staurosporine is a microbial alkaloid that was isolated from *Streptococcus* sp. It inhibits both CDK1 and PKC to induce cell cycle arrest. Staurosporine analogues also exhibit an anti-proliferative effects on various cancer cell lines and this has now entered clinical trials for treating non-small-cell-lung cancer (Tamaoki, 1991, Gani and Engh, 2010).

1.8 CDK inhibitors and CML

Among the above-mentioned CDK inhibitors, flavopiridol (HMR 1275, L86-8275) has entered clinical trials for treatment of CML. Flavopiridal demonstrated potent and specific *in vitro* inhibition of various CDKs (CDK 1, 2, 4 and 7) and inhibits cell cycle progression at the G1/S and G2/M boundaries. In addition, it also inhibits angiogenesis (Rapella et al., 2002) and transcriptional processes through inhibition of the CDK9/cyclin T complex (Chao and Price, 2001).

Flavopiridal induces apoptosis in human leukaemic cells in both *in vitro* and in *vivo* experiments (Decker et al., 2001) and it enhances the activity of imatinib in imatinib-resistant CML cell lines (Yu et al., 2002). Thus, the combination use of flavopiridal and imatinib has entered phase I clinical trials in CML patients and responses are favourable, particularly in imatnib-resistant CML patients (Bose et al., 2012). Moreover, the combination of flavopiridol and other agents, such as the proteasome inhibitor, bortezomib, and microtubule-targeting agent, pyrrolo-1, 5-benzodiazepine, have also been found to induce synergistic effects in inducing apoptosis in imatinib-resistant CML cell lines (Dai et al., 2004, Bright et al., 2010).

1.9 Purvalanol A

Purvalanol A binds between the 2,6,9-trisubstituted purines and the ATP-binding site of the human cyclin dependent kinase 2 (CDK2) (Gray et al., 1998, Gray et al., 1999). This competitive inhibition of ATP binding induces a reversible arrest in the G1 and G2 phases of the cell.

Purvalanol A inhibits the phosphorylation of cyclin dependent kinase substrates, such as Retinoblastoma protein (Rb) and cyclin E. Moreover, it increases the level of the cdk inhibitory protein, $p21^{WAF1/CIP1}$. These effects lead to inhibition of cell proliferation in a range of human cancer cell lines and mouse fibroblasts (Villerbu et al., 2002). Purvalanol A also suppresses Src-mediated transformation of cells. It has been shown that it suppresses c-SRC transformation by inhibiting both cell cycle progression and c-SRC signalling. Therefore, the multi-target, purvalanol A could be a potential inhibitor of cancers that are characterised by c-SRC up-regulation (Hikita et al., 2010).

Recent studies in human cancer cell lines showed that purvalanol A treatment inhibits both CDK2 and the expression of several anti-apoptotic proteins of the Bcl-2 family. Studies in the human gastric adenocarcinoma and aggressive breast cancer cell lines showed that purvalanol A affected both Bcl-2 and Bcl-X_L (Iizuka et al., 2007, OBakan et al., 2014). Furthermore, it can inhibit the phosphorylation at Tyr707 of STAT3 by Janus kinase 2 (JAK2). Therefore, these data suggest that purvalanol A can induce apoptotic cell death by both the down-regulation of anti-apoptotic proteins, and the inhibition of JAK2/STAT3 and polymerase II (Iizuka et al., 2008).

Purvalanol A has now been used in preclinical trials for treating several cancers (Cicenas and Valius, 2011) and so its mechanism of action and effects on drug transporter processes have been investigated. A recent study showed that Purvalanol A is not transported by Breast Cancer Resistance Protein (ABCG2) and P-glycoprotein (ABCB1) (Hofman et al., 2013). In addition, Purvalanol A can inhibit ABCG2 and ABCB1 transporter in ABCG2- and ABCB1- expressing cells, respectively

(Hofman et al., 2013). Thus, this drug is now an interesting candidate for treating efflux transporter-based multidrug resistant tumours.

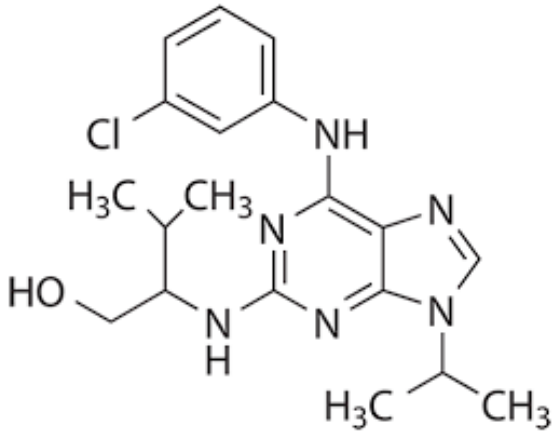

Figure 1.15 Chemical structure of Purvalanol A (Monaco et al., 2004) Purvalanol A is a 2,6,9-trisubstituted purine that inhibits CDK2.

1.10 Summary

CML is a biphasic, myeloproliferative disorder that is associated with expression of the BCR/ABL fusion gene, which encodes a constitutively-active protein tyrosine kinase, leading to a deregulation of tyrosine kinase activity. Although the BCR/ABL tyrosine kinase inhibitor, imatinib, is the frontline therapy for CML, imatinib acquired resistance from mutations in the BCR/ABL gene is a major problem for therapy. Thus, there is a need to find a novel ways to overcome TKI resistance in this disease.

This research project investigates the roles of the protein kinase inhibitor, purvalanol A, in regulating growth and survival in the imatinib-sensitive and imatinib-insensitive CML cell lines, LAMA-84 and KCL-22, respectively.

1.11 Research Aims

The aims of the research are to investigate the effects of purvalanol A on apoptosis of TKI-insensitive CML cells and to elucidate its mechanism of action. The main objectives of this study are:

i) To determine the effects of purvalanol A on apoptosis of TKI-sensitive and -insensitive CML cells.

ii) To investigate the mechanism of action of purvalanol A on CML cells.

iii) To identify the signalling pathways regulated by purvalanol A

iv) To determine the effect of purvalanol A on primary immune cells.

v) To investigate the mechanism of action of JAK3 inhibitor, ZM39923 in CML cells.

1.12 Hypothesis

The main hypothesis to be tested in this project is that "Purvalanol A will provide an adjunct to TKIs in the treatment of CML by inducing apoptosis in TKI-insensitive CML cells".

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

Table 2.1 Materials for neutrophil isolation

Materials	**Supplier**
Lithium heparin vacutainers	Grenier Bio-one (Gloucestershire, UK)
HetaSep™	Stem cell technologies (Cambridge, UK)
Ficoll-Paque™	GE Healthcare (Buckinghamshire, UK)
RPMI 1640 (+25 mM Hepes with L-glutamine)	Gibco (Paisley, UK)
Fetal Bovine Serum	
Human pooled AB serum	Sigma (Poole, UK)
Ammonium chloride lysis buffer Ammonium chloride (NH_4CL) Potassium hydrogen carbonate ($KHCO_3$) Ethylenediaminetetracetic acid (EDTA)	
Isoton	Beckman Coulter Inc. (Florida. USA)

Table 2.2 Materials for CML cell culture

Materials	Supplier
RPMI 1640	Gibco (Paisley, UK)
Foetal Bovine Serum	
Penicillin-Streptomycin	
Isoton	Beckman Coulter Inc. (Florida. USA)
Rapid Romanowsky stain	HD Supplies (Aylesbury , UK)

Table 2.3 Materials for Guava ViaCount assay

Materials	Supplier
Guava ViaCount reagent	Millipore (Hertfordshire, UK)

Table 2.4 Materials for cell cycle analysis

Materials	Supplier
Phosphate buffered saline	Gibco (Paisley, UK)
Propidium iodine	Sigma (Poole, UK)
Ribonuclease A	
Ethanol	
Triton X-100	

Table 2.5 Materials for Annexin V and PI assay

Materials	Supplier
Hanks balanced salt solution	Gibco (Paisley, UK)
FITC-conjugated Annexin V	Invitrogen (Paisley, UK)
Propidium iodide	Sigma (Poole, UK)

Table 2.6 Materials for preparation of protein lysates and Western blotting

Materials	Supplier
Laemmli lysis buffer Glycerol Tris Sodium dodecyl sulphate	Fisher Scientific (Loughborough, UK)
Dithiothreitol	Sigma (Poole, UK)
Bromophenol blue	
Pierce™ BCA protein assay kit	Fisher Scientific (Loughborough, UK)
SDS running buffer Glycine Sodium dodecyl sulphate Tris	Sigma (Poole, UK)
Transfer buffer Tris Glycine Methanol	
Washing buffer Tris Sodium Chloride Tween-20	
Hydrochloric acid	VWR International (Leicestershire, UK)
Stripping buffer Glycine Sodium Chloride	Sigma (Poole, UK)
Phosphate buffer saline tablets	Oxoid Ltd (Basingstoke, UK)
Ammonium persulphate	Sigma (Poole, UK)
Isopropanol	
Tetramethylethylenediamine	
Acrylamide solution	Severn Biotech (Kidderminster,

	UK)
Biotinylated protein ladder detection pack	Cell-Signalling Technology (USA)
Polyvinylidene fluoride membrane	Millipore (Hertfordshire, UK)
Whatman filter paper	Sigma (Poole, UK)
Ponceau S	
Marvel non-fat dry milk	Home Bargain (Liverpool, UK)
Bovine serum albumin	Sigma (Poole, UK)
Immobilon P membrane Chemiluminescence HRP substrate	Millipore (Billerica, UK)
Film developing (developer and fixer)	Millipore (Hertfordshire, UK)
Enhanced chemiluminescence Hyperfilm	Amersham Life Science (Bucks, UK)

Table 2.7 Western blotting antibodies

Materials	**Supplier**
anti-human Mcl-1 #HPA008455	Sigma (Poole, UK)
anti-human Mcl-1 #4572	Cell-Signalling Technology (USA)
anti-human Bcl-2 #2870	
anti-human Bcl-XL #2764	
anti-human Bak #3814	
anti-human p-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (E10) #9106	
anti-human p44/42 MAPK (Erk1/2) #9102	
anti-human p-STAT3 (Tyr705)	

(3E2) #9138	
anti-human STAT3 (124 h6) #9139	
anti-human p-Akt (Ser473) #9271	
anti-human Akt #9272	
anti-human p-NFKB p65 (Ser536) (93 h1) #3033	
anti-human Phospho-p38 MAPK (Thr180/Tyr182) (28B10) #9216	
anti-human p38 MAPK #9212	
anti-human p-Mcl-1 (Ser159/Thr163) #4579	
anti-human β-actin #ab8226	Abcam (Cambridge, UK)

Table 2.8 Material for isolation of mRNA, cDNA synthesis and quantitative PCR

Materials	Supplier
RNeasy kit	Qiagen (Crowley, UK)
DNA digestion solution	
Quantitect SYBR green PCR kit	
Superscript III first strand cDNA synthesis kit	Invitrogen (Paisley, UK)
RNase OUT RNase inhibitor	
Random primers	Promega (Southampton, UK)
Primers	Eurofins (UK)

Table 2.9 Other materials

Materials	Supplier
Imatinib	Cell Signalling (Herts, UK)
Purvalanol A	Tocris Bioscience (Bristol, UK)
ZM39923	
MG-132	
Pan caspase inhibitor	Calbiochem (Darmstadt, Germany)
Cycloheximide	Sigma (Poole, UK)
Phosphatase inhibitor	
JC-1 dye	ThermoFisher Scientific (Waltham, USA)
FCCP	
BIRB796	Selleck Chemicals (Houston, USA)

2.2 Methods

2.2.1 Cell culture

Two BCR-ABL kinase-expressing CML cell lines were used in this study: LAMA-84 (imatinib-sensitive) (Seigneurin et al., 1987, le Coutre et al., 2000) and KCL-22 (imatinib-insensitive) (Kubonishi and Miyoshi, 1983, Quentmeier et al., 2011). Both cell lines were generously provided by Prof. R. E. Clarke, University of Liverpool. Cells were grown at 37ºC in RPMI 1640 medium supplemented with 1 % L-glutamine, 1 % penicillin/streptomycin antibiotics and 10 % Foetal bovine serum (FBS) in a humidified atmosphere containing 5 % CO_2 and maintained in exponential growth phase by routine passaging. Approximately 1 x 10^6 cells in 20 ml media were routinely transferred into 75 cm^2 flasks, as described below.

For passaging, the cell suspension was centrifuged at 500 g for 4 min at 37˚C. The supernatant was discarded and 5 ml of media was added before mixing well. An aliquot was removed and, after a suitable dilution in isoton (balanced electrolytic solution), the cell concentration was measured using a Multisizer 3 cell counter (Beckman coulter). Fresh medium was added so that the final cell density was 5 x 10^4 cells/mL.

For cytospins, cells were diluted into a final concentration of 1 x 10^5 cells/200 µL of phosphate buffered saline (PBS) before being centrifuged at 30 g for 5 min in a Shandon Cytospin3. Cells were then stained with Rapid Romanowsky staining and checked under the light microscope.

Cells were cultured (in the presence and absence of inhibitors) as described in the text, and to avoid mutations, cultures were replenished from frozen stocks every 3 months.

2.2.2 Isolation of primary immune cells from whole blood

The study of adult heathy controls was approved by the University of Liverpool Committee for Research Ethics (CORE). Whole blood was collected into heparinised vacuette containers by venepuncture and processed within 2 h. Neutrophils and PBMCs were isolated by centrifuging over Ficoll-Paque^{TM}, as described in the manufacturer's instructions. Briefly, whole blood was incubated in 5:1 ratio with HetaSep^{TM} for 30 min at 37˚C. The supernatant was collected and diluted in RPMI 1640 media with 25 mM Hepes with L-glutamine (2 mM) before centrifuging at 400 g for 10 min at room temperature. The cell pellet was diluted in media and layered in a 1:1 ratio onto Ficoll-Paque^{TM} before centrifuging at 500 g for 30 min at room temperature. After that, the neutrophils and PBMCs were separated by isosmotic density barrier. The cell bands were then extracted and resuspended into media. The cell suspension was centrifuged at 500 g for 3 min before being resuspended in fresh media. Contaminating erythrocytes were removed by hypotonic lysis (ammonium chloride lysis buffer: 155 mM NH_4Cl, 13.4 mM $KHCO_3$, and 96.7 mM EDTA). The cell media to lysis buffer ratio was 1:9. After 3 min incubation, the cell suspension was centrifuged at 500 g for 3 min. Cells were then resuspended in fresh media and counted before being adjusted a final concentration of 5 x 10^6 cells/mL. The purity of the samples was assessed by light microscopy and cytospins, as described for CML cell lines. Neutrophils and PBMCs were incubated in a 5 % CO_2 incubator at 37ºC with gentle agitation under experimental conditions as described in the text.

2.2.3 Drugs

All drugs used were listed in Table 2.9. All solutions were kept in DMSO at -20˚C.

2.2.4 Flow cytometry for CML cell apoptosis

2.2.4.1 Guava ViaCount assay

Flow cytometry and the ViaCount Assay (Guava Technologies) were used to determine the viability of cells after culture under different conditions. Viability was measured by the permeability of the plasma membrane to DNA-staining dyes.

25 μL of cell suspension was added to 225 μL of Guava Viacount reagent in each well of a 96-well plate. The contents of each well was then analysed on the flow cytometer using Guava software. 5,000 gated cells were measured and the percentages and numbers of viable cells were calculated for each sample.

2.2.4.2 Cell cycle analysis

The cell cycle kinetics assay measures the amount of the DNA per cell using flow cytometry by DNA staining of permeabilised cells with propidium iodide (PI). The DNA content per cell is related to the position of the cell in the cell cycle.

200 μL of cell suspension were centrifuged at 1000 g for 4 min and washed in 200 μL of PBS. Cells were then fixed in 200 μl of 70 % ethanol (which also increases plasma membrane permeability) for at least 2 h, before being washed twice and resuspended in 200 μL of PBS in a well of a 96 well plate. 25 μl of PI solution was added (containing 1 mg/mL RibonucleaseA in 1 % Triton/PBS, final PI concentration of 100 μg/mL), and incubated for at least 1 h (in the dark) before analysing by flow cytometry. The data were then analysed using Guava Cell Cycle software to calculate the percentage of cells in different phases of the cell cycle (G0/Apoptotic, G1, G2 and S).

2.2.5 Flow cytometry to determine primary immune cell apoptosis

Apoptosis of neutrophils and PBMCs was measured by flow cytometry. Phosphatidylserine expressed on the membrane of apoptotic cells was stained by Annexin V-FITC, while DNA was stained by PI in necrotic cells. Cells at a concentration of 2 x 10^5 in 200 µL HBSS were incubated with Annexin V-FITC (10 µL/mL) for 15 min at room temperature. Prior to the flow analysis, Propidium Iodide (final concentration of 100 µg/mL) was added to the cells. 5,000 gated cells were then analysed by the Guava Easycyte flow cytometer.

In all experiments using the flow cytometer, the absolute number of cells was routinely counted in order to avoid misinterpretation due to lysed cells. The % and actual number of cells were routinely measured and compared.

2.2.6 Flow cytometry to determine mitochondrial depolarisation

Flow cytometry was used to measure changes in polarisation of the inner mitochondrial membrane by JC-1 fluorescence after incubation under different conditions. After incubation, suspensions containing 10^6 cells were pipetted into a 1.5 ml microfuge tube and centrifuged at 2000 g for 3 min. The pellet was resuspended in 1 mL HBSS, 1 µl JC-1 dye (Molecular Probes) added (final concentration of 1 µg/mL) and incubated at 37˚C for 15 min in the dark. 25 µl of cell suspension were removed and mixed with 225 µl HBSS in a 96 well plate and analysed by flow cytometry. As a positive control to show mitochondrial membrane depolarisation, one well was treated with 1 µl FCCP (final concentration 1 µg/mL) and incubated at 37˚C for further 15 min before analysis. Membrane depolarization was measured as a red to green shift in fluorescence.

2.2.7 Preparation of protein lysates

10^6 CML cells per sample were centrifuged and washed with PBS to obtain cell pellets, which were resuspended in 100 µL hot Laemmli lysis buffer (10 % (v/v) glycerol, 1 M Tris-HCl (pH 6.8), and 3 % (w/v) SDS),

to a final concentration of 1 x 10^4 cells/μL before boiling for 5 min. All protein extract samples were then analysed for total protein levels by the bicinchoninic acid (BCA) assay and adjusted to achieve the same protein concentration. The BCA assay is a detergent-compatible formulation for the colorimetric detection and quantitation of proteins. The principle of BCA is based on a chelation of copper with protein in an alkaline environment. Thus, BCA binds to the reduced cuprous cation (Cu^{1+}) and exhibits a strong linear absorbance at 562 nm (Smith, et al. 1985).

5 μL of cell lysate from each sample was incubated with 195 μL of BCA solution (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide with 4 % cupric sulfate) in a 96-well plate for 30 min in a 5 % CO_2 incubator at 37ºC with gentle agitation. Then, the plates were analysed on a plate reader at 562 nm, before adjusting the samples to achieve equal concentrations by adding Laemmli lysis buffer, DTT, and bromophenol blue. Samples were then stored at -20ºC until analysis.

2.2.8 Western blotting

Thawed protein extract samples were re-heated and separated by SDS-PAGE on a 8-12% resolving gel, depending on the relative molecular mass of the proteins to be resolved. The resolving gels were composed of 8-12 % (v/v) polyacrylamide, 1 % (w/v) SDS, 370 mM Tris-HCl (pH 8.8), 1 % (w/v) APS and 0.1 % (v/v) TEMED. To induce polymerization, APS and TEMED were added last. The stacking gels were composed of 4.5 % (v/v) polyacrylamide, 1 % (v/v) SDS, 122 mM Tris-HCl (pH 6.8), 1 % (w/v) APS and 0.1 % (v/v) TEMED. 15 μl of protein extract was added to each well of the gel, with pre-stained marker proteins loaded in the first well. The gel was electrophoresed at 180 V for 55 min in SDS running buffer (25 mM Tris, 0.1 % (w/v) SDS, and 192 mM glycine) using a Bio-Rad Mini Protean II Electrophoresis kit. Then, separated proteins were transferred onto a PVDF membrane by electrophoresis at 100 V for 80 min in transfer buffer (20 % (v/v) methanol, 12.5 mM Tris, and 95 mM glycine) using the same apparatus. The membrane was checked to determine if the proteins

had been successfully transferred onto the membrane, by immersing into Ponceau S stain (0.1 % (w/v) Ponceau S and 5 % (v/v) acetic acid). After washing off the Ponceau stain by washing buffer (150 mM NaCl, 10 mM Tris HCl (pH 8), and 0.1 % (v/v) Tween-20), the membrane was blocked by blocking solution (5 % non-fat dried skim milk (Marvel) in TBS-T (0.05 % Tween 20 in Tris-buffered saline, pH 8.0)) at room temperature for at least 1 h on an orbital shaker in order to prevent non-specific antibody binding. After blocking, the membrane was rinsed with washing buffer for 30 sec and then incubated with the specific primary antibody (dilutions as specified by manufacturer's datasheet, Table 2.10) at 4°C overnight on an orbital shaker.

The following day, the membrane was washed with wash buffer for 3 x 10 min and then incubated with secondary horseradish peroxidase (HRP)-linked antibody for 1 h on the same apparatus. After washing again by wash buffer for 3 x 10 min, the membrane was covered with Enhanced Chemiluminescence (ECL) reagents for 1 min and then exposed to photosensitive film for periods of 1-10 min depending on intensity of the signal, taking care to avoid saturation of the film.

After developing the blot, the membrane was stripped by immersing in stripping buffer (50mM glycine, 150 mM NaCl, 0.1 % (v/v) Tween-20, and HCl to pH 2.5) for 15 min with gentle agitation and then washed. The membrane was re-blocked and re-probed with actin antibody to ensure equal loading of wells. The membrane was then developed as indicated above. The exposed film was then analysed by AQM advance 6 Kinetic Imaging System. Table 2.10 summarises antibodies used for western blotting.

Table 2.10 Western blot antibodies

Protein of interest	**Primary antibody**	**Secondary antibody**
Mcl-1	Rabbit anti-human Mcl-1 (1:1,000)	HRP-conjugated donkey anti-rabbit IgG (1:10,000)
Bcl-2	Rabbit anti-human Bcl-2 (1:1,000)	HRP-conjugated donkey anti-rabbit IgG (1:10,000)
Bcl-X_L	Rabbit anti-human Bcl-X_L (1:1,000)	HRP-conjugated donkey anti-rabbit IgG (1:10,000)
Bak	Rabbit anti-human Bak (1:1,000)	HRP-conjugated donkey anti-rabbit IgG (1:10,000)
Phospho-Erk	Mouse anti-human phospho-Erk (1:2,000)	HRP-conjugated sheep anti-mouse IgG (1:10,000)
Erk	Rabbit anti-human Erk (1:1,000)	HRP-conjugated donkey anti-rabbit IgG (1:10,000)
Phospho-STAT3	Mouse anti-human phospho-STAT 3 (1:1,000)	HRP-conjugated sheep anti-mouse IgG (1:10,000)
STAT 3	Rabbit anti-human STAT 3 (1:2,000)	HRP-conjugated donkey anti-rabbit IgG (1:10,000)
Phospho-Akt	Rabbit anti-human phospho-Akt (1:1,000)	HRP-conjugated donkey anti-rabbit IgG (1:10,000)
Akt	Rabbit anti-human Akt (1:1,000)	HRP-conjugated donkey anti-rabbit IgG (1:10,000)
Phospho-p38	Mouse anti-human	HRP-conjugated sheep

	phospho-p38 (1:2,000)	anti-mouse IgG (1:10,000)
p38	Rabbit anti-human p38 (1:1,000)	HRP-conjugated donkey anti-rabbit IgG (1:10,000)
Phospho-Mcl-1	Rabbit anti-human p38 (1:1,000)	HRP-conjugated donkey anti-rabbit IgG (1:10,000)
β-actin	Mouse anti-human β-actin (1:10,000)	HRP-conjugated sheep anti-mouse IgG (1:10,000)

2.2.9 Isolation of CML cells mRNA

Total mRNA of CML cells was isolated using the RNeasy kit (Qiagen), according to the manufacturer's instructions. First, 10^6 CML cells were centrifuged at 1,000 g for 3 min to obtain a cell pellet. The cell pellet was disrupted using 350 µL of a highly denaturing guanidine-thiocyanate-containing buffer (Buffer RLT) with 3.5 µL β-mecaptoethanol (to inactivate RNases) before being homogenised by a syringe and needle. Cell lysates were passed through a 20-guage needle 5 times or until the lysate became homogeneous. Then, 350 µL ethanol (70 % (v/v) in DEPC-treated water) was added to create conditions that promote selective binding of RNA to a RNeasy silica-membrane. After that, 700 µL of sample was transferred to RNase Mini spin column. Each column was then centrifuged at 8,000 g for 15 sec and the flow-through discarded before the column was washed with 350 µL RW1 buffer by centrifuging at 8,000 g for 15 sec. Residual DNA was removed from the column by DNA digestion using RNase-Free DNase. The column was treated by DNase I (10 µL DNase (2.7 units/µL) with 70 µL RDD buffer) for 15 min at room temperature and washed with 350 µL RW1 buffer (centrifuging at 8,000 g for 15 sec). The column was then washed twice with 500 µL RPE buffer by centrifuging at 8,000 g for 15 sec and 2 min, respectively.

The column was then placed into a 2 ml collection tube and centrifuged at the full speed for 1 min to get rid of excess buffer. Then, the column was transferred to a new 2ml collection tube. 30 µL RNase-free water was directly added to the membrane before being centrifuged at 8,000 g for 1 min in order to elute RNA in RNase-free water.

The quantity and quality of RNA obtained was measured using a Nanodrop ND-100 spectophotometer. The RNA samples were then stored at -80˚C.

2.2.10 cDNA synthesis

Complementary DNA was converted from total mRNA using the Superscript III First Strand Synthesis Kit and RNAse OUT (Qiagen), according to the manufacturer's instructions. The amount of RNA in each sample was adjusted between samples within each experiment with RNase–free water to a concentration of 200-250 ng. Each RNA sample was added to 1 µL (250 ng or 20 µM) random primer, 1 µL (10 mM) dNTPs and RNase-free water to a total volume of 13 µL. The samples were then heated in a thermo PX2 thermal cycler at 65˚C for 5 min before cooling on ice for at least 1 min. A mixture of 4 µL First-Strand buffer, 1 µL (0.1 M) DTT, 1 µL RNase OUT (RNase inhibitor), and 1 µL (200 units/µL) Superscript III reverse transcriptase were added to each sample. Each sample was then incubated 25˚C for 5 min for initiation, 50 ˚C for 60 min, and 70˚C for 15 min. The synthesized cDNA was then stored at -20˚C. Table 2.11 summarises the cDNA synthesis procedure.

Table 2.11 cDNA synthesis procedure

Reagents	Volume (µL)	Concentration	Temp (˚C)	Time (min)
RNA (with RNase-free water)	11	200-250 ng/µL	60	5
Random primer	1	20 µM	On ice	>1
dNTPs	1	10 mM		
First-Strand buffer	4	5X	25	5
DTT	1	0.1 M		
RNase OUT	1	40 units/ µL	50	60
Superscript III reverse transcriptase	1	200 units/ µL	70	15
Total volume	20			

2.2.11 SYBR based Quantitative Polymerase Chain Reaction

SYBR based Quantitative Polymerase Chain Reaction (qPCR), was used to amplify and simultaneously detect and quantify amplified cDNA molecules using appropriate forward and reverse primers by qPCR. After converting mRNA into cDNA, cDNA was quantified by a thermal cycler (Light cycler) using the QuantiTect SYBR green detection kit (Qiagen), according to manufacturer's instructions. 1 µL cDNA was added to a mixture of 10 µL Quantitect and 0.8 µL (10 pM) of each forward and reverse primer, and RNase free-water to a 20 µL of total reaction volume. Each sample was prepared in duplicate and qPCR was performed using a Roche LightCycler 480 qPCR machine. The level of gene expression of target gene (e.g. Mcl-1) was normalised to β-Actin by comparing the Ct value, according to the Pfaffl method (Pfaffl, 2001). Table 2.12 and 2.13 summarise the primer sequences and quantitative PCR cycle setting protocols, respectively.

Table 2.12 Gene and Primers for qPCR

Gene	**Base pair length**	**Forward primer 5'-3'**	**Reverse primer 3'-5'**
Mcl-1L	170	TTATCTCTCGGTACCTCCGG	GATGTCCAGTTTCCGAAGCA
β-Actin	211	CATCGAGCACGGCATCGTCA	TAGCACAGCCTGGATAGCAAC

Table 2.13 qPCR cycling parameters

Step	**Temp (˚C)**	**Time**	**Number of cycles**	**Analysis Mode**
1. Taq activation	95	15 min	1	None
2. Denaturation Annealing Elongation	94 55 72	1 min 30 sec 30 sec	45	Quantification
3. Generating melt curves and checking product specificity	60	30 sec	1	Melting Curves
4. Cooling	40	10 sec	1	None

2.2.12 Statistics

All data sets are expressed as mean ± SEM, and differences are considered significant for p values of ≤ 0.05. Comparisons are made using a parametric statistical analyses, as specified and as appropriate to the data sets. Paired two-tailed Student's t-test or One-way ANOVA followed by *post hoc* comparisons tests were used for normally distributed data. Error bars, representing SEM, are shown in most of figures, but due to the low error in some datasets some error bars may appear extremely small or undetectable.

CHAPTER 3: EFFECTS OF IMATINIB ON IMATINIB-SENSITIVE AND -INSENSITIVE CML CELL LINES

3.1 Introduction

CML results in the increased and unregulated growth of predominantly myeloid cells in the bone marrow and the accumulation of these cells in the blood. It is associated with a single genetic abnormality known as the Philadelphia (Ph) chromosome which results from a t(9;22) reciprocal translocation to generate the BCR/ABL fusion gene. This gene encodes a constitutively-active protein tyrosine kinase, leading to a deregulation of tyrosine kinase activity within CML cells. This results in the activation of pathways associated with cell proliferation, control of apoptosis, differentiation, and adhesion, which are all implicated in CML pathology (Ren, 2005, Deininger et al., 2000, Kurzrock et al., 1988, Quintas-Cardama and Cortes, 2009).

The BCR/ABL tyrosine kinase inhibitor (TKI), imatinib, which binds directly to the adenosine triphosphate-binding site of BCR-ABL kinase, was developed as a frontline therapy for CML (Nagar et al., 2002). Imatinib increases the average life expectancy of CML patients from the 4-6 years obtained with interferon-based treatment (Sacchi et al., 1997) to a survival rate of decades (Smith et al., 2010). It has been reported that imatinib can induce complete cytogenetic response in almost 80% of patients diagnosed with CML chronic phase with mild toxicity (Kantarjian et al., 2002) and more than 70 % of patients may achieve a major molecular response by 5 years of therapy (Kantarjian et al., 2008).

However, imatinib-acquired resistance occurs and this remains an obstacle to eradicate this disease. The most common mechanism that results in acquired resistance is mutation of the BCR/ABL kinase domain that affects its binding with imatinib (Soverini et al., 2014, Branford et al., 2003), while other mechanisms, such as BCR-ABL amplification and

alterations in drug influx and efflux mechanisms are also found (Mahon et al., 2000, Ni et al., 2011, Gorre et al., 2001). While the introduction of second- and third-generation TKIs such as nilotinib, dasatinib, and ponatinib can overcome much of this imatinib-resistance, the high rate of mutations that occur in CML cells also result in the development of resistance to these new drugs. (An et al., 2010, Kantarjian et al., 2006, Talpaz et al., 2006, Zabriskie et al., 2014). As a result of this development of resistance to TKIs, there is a need to find alternative ways to kill CML cells by other kinase inhibitors that may induce apoptosis in TKI-resistant CML cells.

Immortalised cell lines derived from patients who develop malignancies are an important tool to understand cancer biology (Drexler et al., 2000). Thus, the use of such cell lines in leukaemia research can help understand disease mechanisms and test new therapeutic approaches (Drexler and MacLeod, 2002, Drexler and Matsuo, 2000).

There are several BCR-ABL-positive cell lines that have been used in CML studies. These cell lines include LAMA-84, EM3, K562, KYO1, KU812, KCL-22, BV173, AR230, TOM1 and SD1. Most of them were derived from CML patients in blast crisis (Mahon et al., 2000), while two of them, TOM1 and SD1 were derived from BCR-ABL-positive acute lymphocytic leukaemia patients (Okabe et al., 1987, Dhut et al., 1991). Previous research has shown that imatinib suppressed the growth of most of BCR-ABL-positive cell lines. However, there are two cell lines, KCL-22 and SD1, which demonstrate natural resistance to imatinib (Deininger et al., 1997).

LAMA-84 cell line was derived from the peripheral blood of a 29-year old CML patient who had been treated with busulfan for 5 consecutive years. The cells were collected one month after the onset of blast crisis. These cells are reported to express BCR-ABL and respond to several agents including imatinib (Blom et al., 1996, Seigneurin et al., 1987).

KCL-22 cell line was established from the plural effusion of a 32-year old CML patient in blast crisis. These cells are described to contain

BCR-ABL (Kubonishi and Miyoshi, 1983). The KCL-22 cell line was found to be insensitive to imatinib despite its expression of wild type BCR-ABL (Deininger et al., 1997).

Thus, these cell lines could be potential *in vitro* models to determine the mechanisms of imatinib resistance. In addition, they have been previously shown in this laboratory to have different sensitivity to imatinib (Guest, 2012) LAMA-84 cells, are imatinib-sensitive and KCL-22 cells have greater resistance to imatinib. Therefore, LAMA-84 and KCL-22 cell lines were selected in this study to represent as imatinib-sensitive and –insensitive CML cell lines, respectively.

The aims of the work described in this Chapter were:

i) To determine the effects of imatinib on the growth and apoptosis of these two cell lines.

ii) To measure the expression of Bcl-2 family members in these two cell lines and to determine how the levels of these proteins are affected by imatinib.

3.2 Methods

All methods described in this Chapter are more fully detailed in Chapter 2. Imatinib was used at 10 μM (unless otherwise stated) which was shown in previous studies in this laboratory (Guest, 2012, Goodman, 2012) to induce maximal effects on the growth of these cells and is the serum concentration that is obtained therapeutically during the treatment of CML patients (Rezende et al., 2013). As imatinib was dissolved in DMSO, all control experiments contained equivalent amounts of this solvent (typically 0.1 %, v/v unless stated otherwise). In experiments using imatinib plus other compounds dissolved in DMSO, the maximal DMSO used in experiments was 0.3 % (v/v) and this concentration did not have any measured effect of any of the cell parameters analysed.

3.3 Results

Initial experiments measured the effects of imatinib on apoptosis of these cell lines, which was measured in the following ways: (a) using the Viacount assay, which measures changes in the plasma membrane permeability to DNA-binding dyes; (b) measuring cell cycle kinetics, by measuring the proportion of cells in each of the cell cycle phases, including G0 (measuring the decreased DNA content of apoptotic cells); (c) using JC-1 dye, which measures changes in mitochondrial inner membrane potential, which becomes depolarised during the initiation of apoptosis and detected as a shift in JC-1 fluorescence from red to green; (d) using western blotting to detect changes in Bcl-2 family protein expression of pro- and anti-apoptotic proteins, such as Mcl-1, Bcl-2, Bcl-X_L, and Bak; (e) using the pan-caspase inhibitor, Z-VAD which blocks the function of activated caspases and hence prevents the development of apoptotic cell features that occur via caspase-dependent processes.

3.3.1 Effects of imatinib on the viability of LAMA-84 and KCL-22 cell lines

LAMA-84 and KCL-22 cells were initially tested for their sensitivity to different concentrations of imatinib, in terms of the ability of this drug to induce changes in viability. LAMA-84 cells and KCL-22 cells were incubated for 24 h in the presence and absence of gradient concentrations of imatinib (2.5, 5, 10, and 15 µM) and viability was assessed using the ViaCount assay.

These two cell lines showed significantly different sensitivities to these concentrations of imatinib ($P \leq 0.001$, two-way ANOVA with replication), as shown in Figure 3.1. At a concentration of 15 µM imatinib, KCL-22 cells showed only less than 10 % decrease in viability, while LAMA-84 cells showed more than 30 % loss of viability. The results show that imatinib at 10 µM induced apoptosis significantly in LAMA-84 cells within 24 h of incubation, from 92.7 ± 0.5 % viability of control samples to 61.7 ± 1 % viability of imatinib treated samples ($p \leq 0.01$, n=3). However, under these experimental conditions, this drug had no significant effect on the viability of KCL-22 cells (control 92.4 ± 0.6 % viability of control samples and 89.4 ± 0.4 % viability of imatinib treated samples) ($p > 0.05$, n=3).

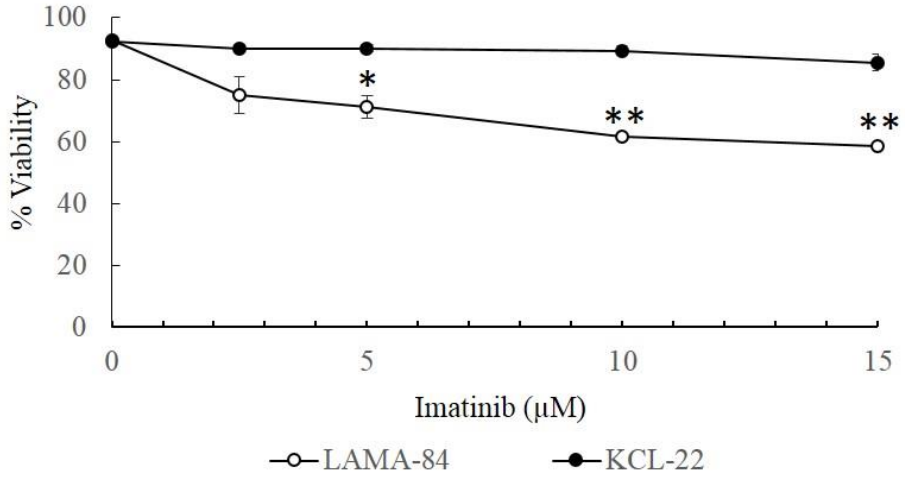

Figure 3.1 Differential sensitivity of LAMA-84 and KCL-22 cell lines to imatinib. Viability was determined using the Viacount assay, in control cells or cells incubated with gradient concentrations of imatinib, as follows: LAMA-84 cell line ●; KCL-22 cell line ○. Data are shown as mean (±SEM, n=3) *= p≤0.05, **=p≤0.01 (paired two-tailed student's t-test).

3.3.2 Effects of imatinib on cell number and morphology of LAMA-84 and KCL-22 cell lines

The numbers of LAMA-84 and KCL-22 cells after 24 h incubation in the absence (control) and presence of imatinib, were counted by flow cytometry, while cell morphology was evaluated by light microscopy. These combined parameters were used to confirm apoptosis of CML cells by appearance of apoptotic morphology.

Imatinib significantly decreased the number of LAMA-84 cells (from 2.2 x 10^6 ± 1.7 x10^5 cells/ml in control samples to 1.8 x 10^6 ± 1.1 x 10^5 cells/ml in imatinib treated suspensions), ($p \leq 0.05$, n=3), but it had no significant effect on the number of KCL-22 cells counted (average 2.2 x 10^6 ± 1.3 x 10^5 cells/ml of control samples to 2 x 10^6 ± 3.3 x10^4 cells/ml in imatinib treated samples), ($p > 0.05$, n=3) (Figure 3.2). These results correlate with the viability change measured (using the Viacount assay) after treating cells with imatinib.

After counting cell numbers, cell samples were stained and visualised by light microscopy (see Materials and Methods). These results confirmed that imatinib induced apoptosis in LAMA-84 cell, but had little effect on KCL-22 cells. After treatment of LAMA-84 cells with imatinib, the cells were smaller and showed condensed chromatin (cells in Figure 3.3, particularly inset at high magnification) In contrast, cells with this morphology were not frequently found in KCL-22 cells treated with imatinib, compared to LAMA-84 cells (Figure 3.3).

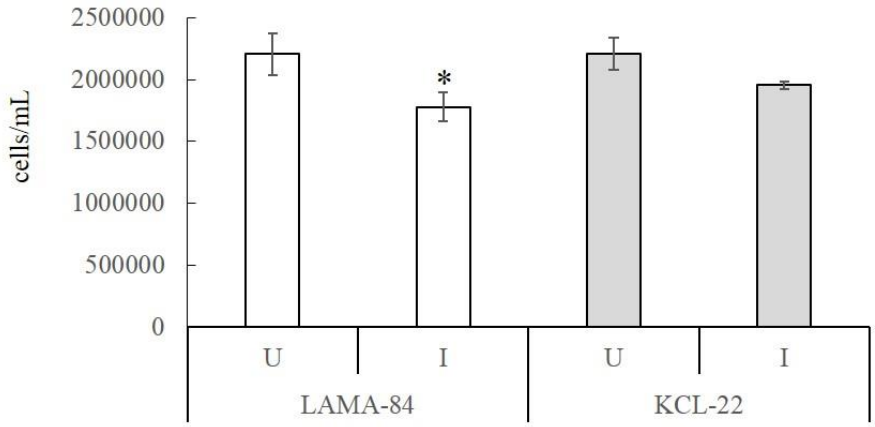

Figure 3.2 Effect of imatinib on the number of LAMA-84 and KCL-22 cells. Both LAMA-84 and KCL-22 cells were incubated for 24 h in the absence (U) and presence (I) of imatinib (10 μM). Total cell counts were assessed by flow cytometry using Viacount reagent (see Materials and Methods). Data are shown as mean (±SEM, n=3) *=$p \leq 0.05$ (paired two-tailed student's t-test).

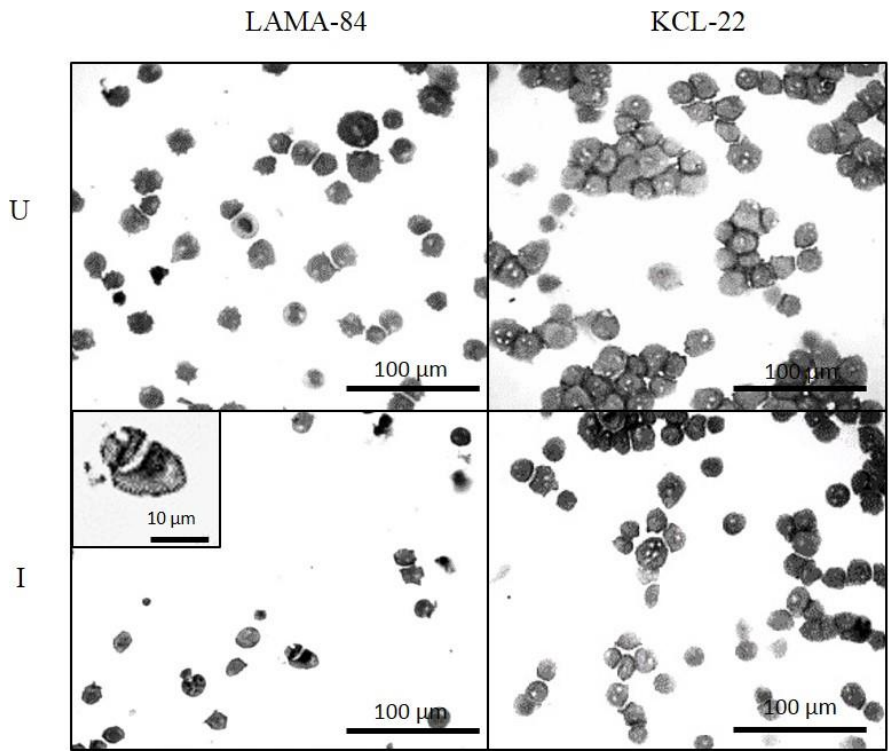

Figure 3.3 Effect of imatinib on LAMA-84 and KCL-22 cell morphology. Both LAMA-84 and KCL-22 cells were incubated for 24 h in the absence (U) and presence (I) of imatinib (10 µM). Cell morphology was observed by light microscopy after staining of cytospins (see Materials and Methods).

3.3.3 Effects of imatinib on cell cycle kinetics of LAMA-84 and KCL-22 cell lines

The effects of imatinib on the growth parameters of LAMA-84 and KCL-22 cells were also measured by cell cycle analysis. LAMA-84 cells and KCL-22 cells were incubated for 24 h in the presence and absence of gradient concentrations of imatinib (2.5, 5, 10, and 15 µM) prior to measuring cell cycle kinetics by analysis of DNA content.

As shown in Figure 3.4, imatinib treatment caused a significant increase in the number of cells in the G0 phase (low DNA content per cell, or apoptotic cells) of LAMA-84 cells, but not in KCL-22 cells. 24 h incubation of 10 µM imatinib in LAMA-84 cells induced 46.4 ± 1.9 % of the total cell population to accumulate in G0. This was significantly increased compared to untreated cells (14.4 ± 1.5 % of total cell population, $p \leq 0.01$, n=3) (Figure 3.4A) and confirmed the results obtained with the Viacount assay that this agent induced apoptosis in these cells (Section 3.3.1).

In contrast, 10 µM imatinib did not cause a significant increase in the G0 population of KCL-22 cells, but instead, this drug induced a partial cell cycle arrest in G1 (59.2 ± 3.4 % of total cell population in G1 of imatinib treated samples compared to 46.1 ± 1.1 % of untreated samples, $p \leq 0.05$, n=3), with fewer cells accumulating in G2 (results not shown) after drug treatment (Figure 3.4B).

In summary, cell cycle analysis confirmed an induction of apoptosis in LAMA-84 cells by imatinib but additionally revealed a G1 arrest of KCL-22 cells.

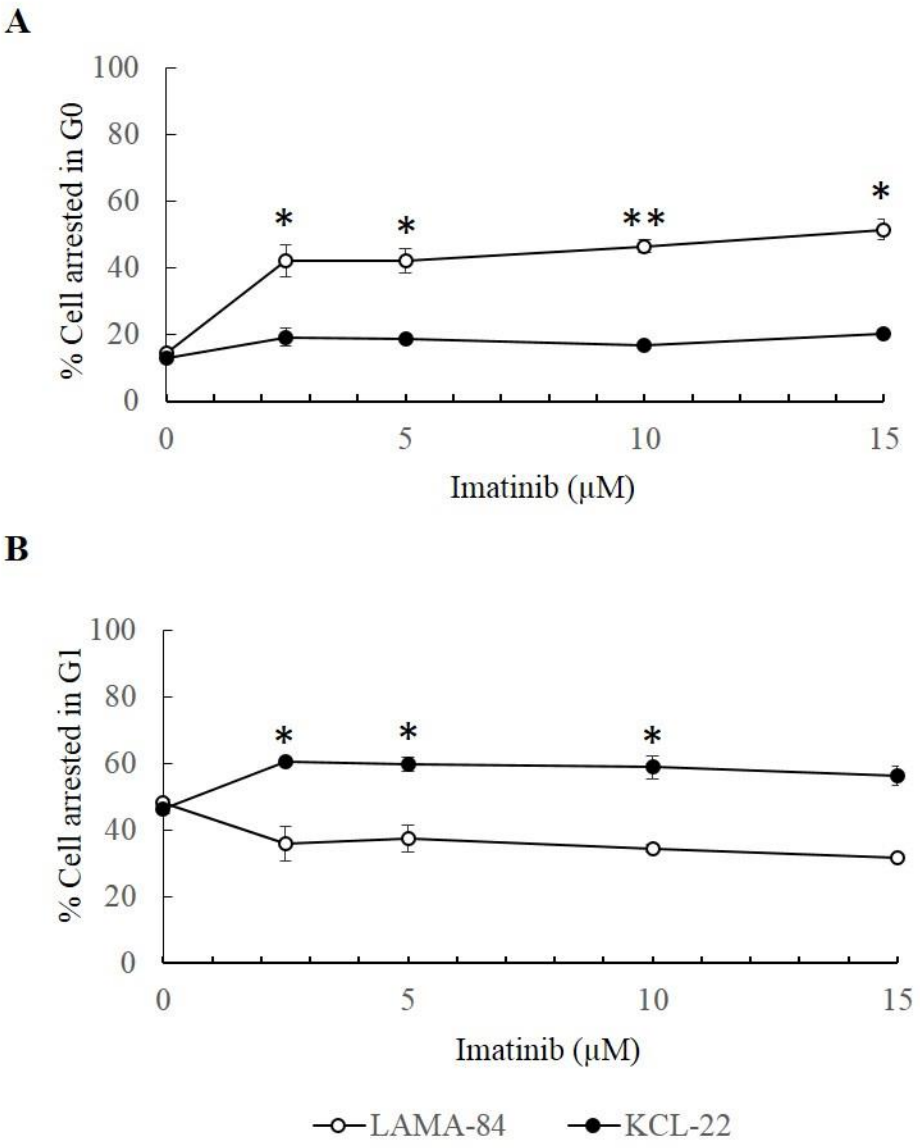

Figure 3.4 Effect of imatinib on LAMA-84 and KCL-22 cell cycle kinetics. Cell cycle parameters (expressed as a percentage of the total cell population) of cells arrested in G0 **(A)** and G1 **(B)** were determined by measuring DNA content and flow cytometry. Data are shown as mean (±SEM, n=3) *=p≤0.05, **=p≤0.01 (paired two-tailed student's t-test).

3.3.4 Effects of imatinib on mitochondrial polarisation of LAMA-84 and KCL-22 cell lines

LAMA-84 and KCL-22 cells were incubated in the presence and absence of 10 μM imatinib and then samples were collected at time points of 0 h, 30 min, and 1, 2, 4, 6, 18 and 24 h. They were then incubated with JC-1 dye before measuring the fluorescence shift by flow cytometry (see Materials and Methods). JC-1 binds to mitochondrial membranes and exhibits red fluorescence when the membrane is highly polarised, but green fluorescence when the inner mitochondrial membrane becomes depolarised during the early stages of induction of apoptosis.

In untreated cells, no membrane depolarization was detected in either cell line at any time point. However, imatinib induced mitochondrial membrane depolarisation in LAMA-84 cells, but not in KCL-22 cells, as shown in Figure 3.5. Imatinib caused significant mitochondrial depolarisation in LAMA-84 cells within 4 h from 4.2 ± 0.3 % of 0 h to 13.9 ± 0.2 % by 4 h of imatinib incubation, ($p \leq 0.01$, $n=3$). This indicates that imatinib caused rapid changes in mitochondrial function that may be attributed to the induction of apoptosis.

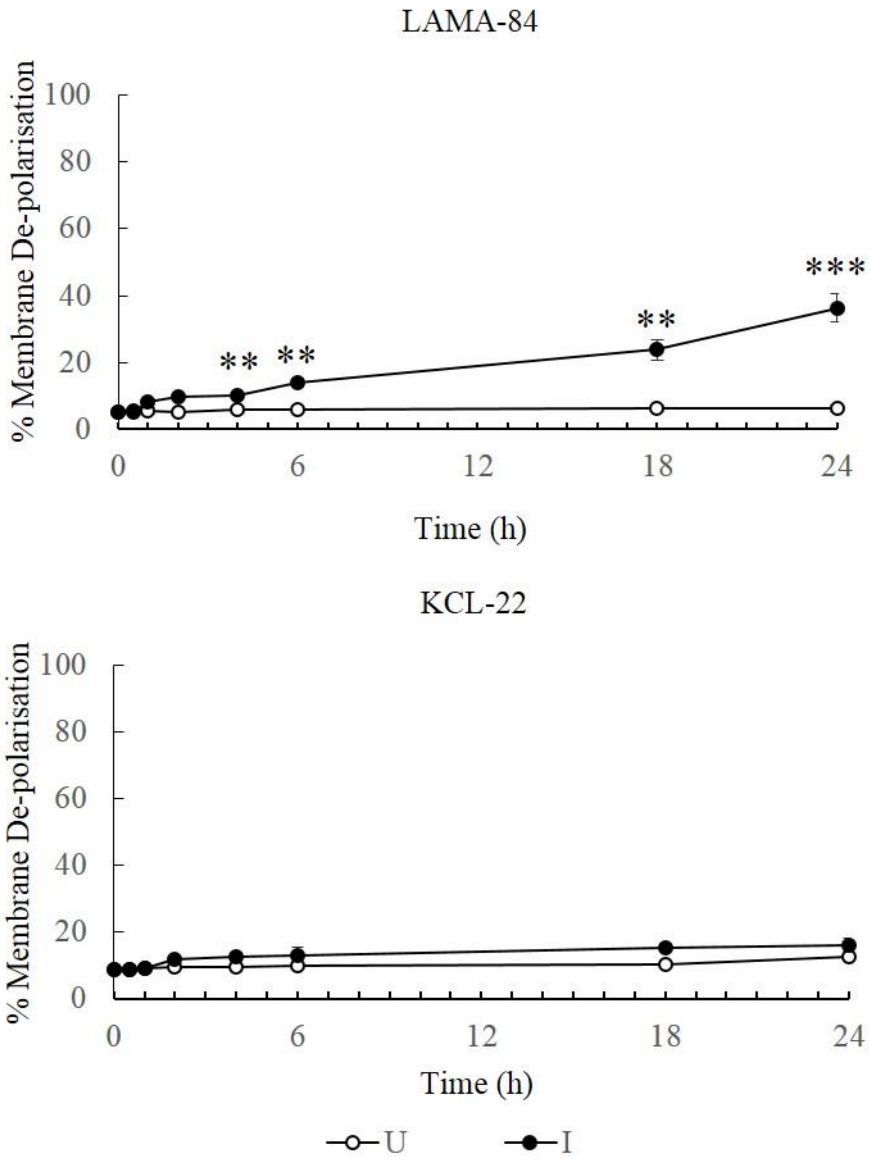

Figure 3.5 Effects of Imatinib on mitochondrial polarisation in LAMA-84 and KCL-22 cells. Both LAMA-84 and KCL-22 cell lines were incubated in the absence (U ○) and presence of imatinib (10 μM) (I ●). The samples were collected at various time points after addition of drugs. Mitochondrial polarisation (expressed as a percentage of the total cell population) was determined by flow cytometry as a shift from red fluorescence to green fluorescence (see Materials and Methods). Data are shown as mean (±SEM, n=3) **=p≤0.01, ***=p≤0.001, compared to 0 h time point (One-way ANOVA followed by *Bonferroni post hoc* comparisons tests).

3.3.5 Effects of imatinib on expression of anti-apoptotic Bcl-2 family proteins in LAMA-84 and KCL-22 cell lines

Western blotting was used to determine the effects of imatinib on the expression levels of Bcl-2 family proteins in both LAMA-84 and KCL-22 cell lines. Both cell lines were treated with and without imatinib (10 μM) for 24 h and then protein lysates collected for western blotting.

3.3.5.1 Anti-apoptotic protein, Bcl-2

Imatinib resulted in a small decrease in Bcl-2 expression in LAMA-84 cells, but these small changes were not significantly different to control cell values. In contrast, KCL-22 cells did not express Bcl-2 protein (Figure 3.6). This latter experiments were repeated a number of times with different anti-Bcl-2 antibodies and similar results were obtained. Thus, it can be concluded that KCL-22 cells do not express Bcl-2 protein.

3.3.5.2 Anti-apoptotic protein, Bcl-X_L

Imatinib resulted in a small decrease in expression levels of Bcl-X_L levels in both LAMA-84 and KCL-22 cells, but these decreases did not reach statistical significance compared to untreated values (Figure 3.6).

3.3.5.3 Pro-apoptotic protein, Bak

Imatinib resulted in a small increase in Bak expression in both cell lines, but these small changes in expression values did not reach statistical significance (Figure 3.6).

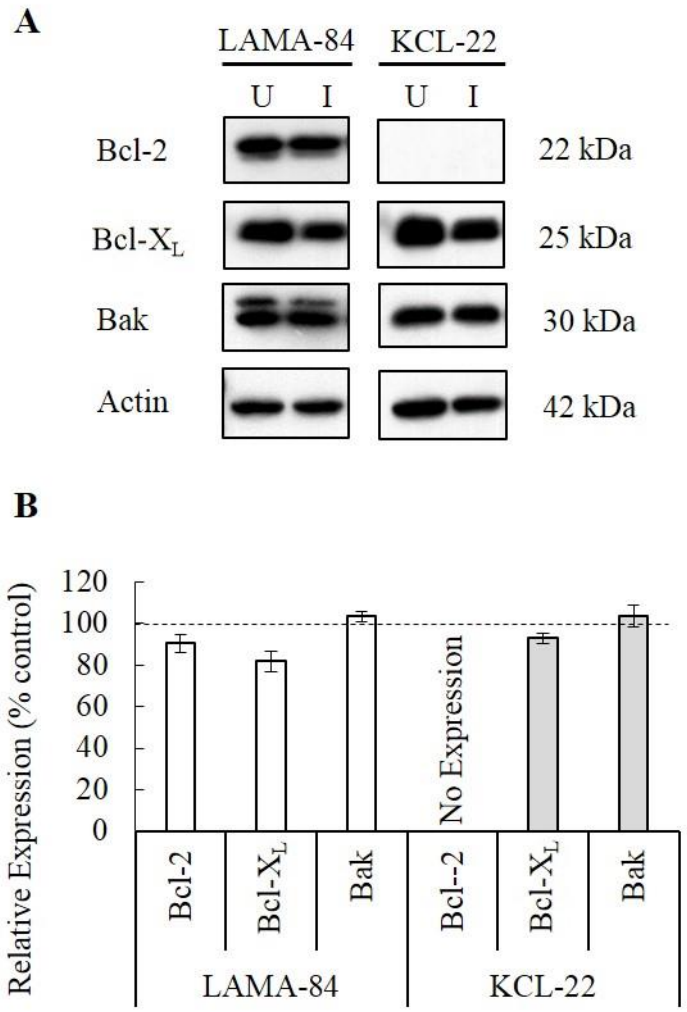

Figure 3.6 Effects of imatinib on expression of Bcl-2, Bcl-X_L and Bak in LAMA-84 cells and KCL-22 cells. Cells were incubated in the absence (U) or presence (I) of 10 µM imatinib for 24 h and protein extracts were analysed by western blotting. **(A)** shows examples of western blots for Bcl-2 (22 kDa), Bcl-X_L (25 kDa), Bak (30 kDa) and actin (42 kDa). **(B)** shows densitometric analysis of the effects of imatinib on Bcl-2, Bcl-X_L and Bak expression, normalised for actin levels and expressed as a % of untreated values (±SEM, n=3). Western blot shown is representative from n=3 experiments.

3.3.5.4 Anti-apoptotic protein, Mcl-1

In LAMA-84 cells, imatinib resulted in a significant decrease of Mcl-1 protein expression. Densitometric analysis indicated that Mcl-1 expression was decreased significantly from 100% of untreated samples to 51.5 ± 6.7 % of imatinib treated samples ($p \leq 0.05$, n=3) (Figure 3.7B). In contrast, imatinib had no significant effect on Mcl-1 expression in KCL-22 cells. After 24 h of imatinib incubation, the relative expression of Mcl-1 in KCL-22 cells was 98.8 ± 9 % in the presence of imatinib, compared 100 % in control cells (Figure 3.7B).

In conclusion, imatinib caused no significant changes in expression of Bcl-2, Bcl-X_L, and Bak in both cell lines, but caused a significant decrease in expression of the anti-apoptotic protein, Mcl-1 in LAMA-84 cells, which is sensitive to imatinib. In addition, the KCL-22 cell line does not express Bcl-2 protein.

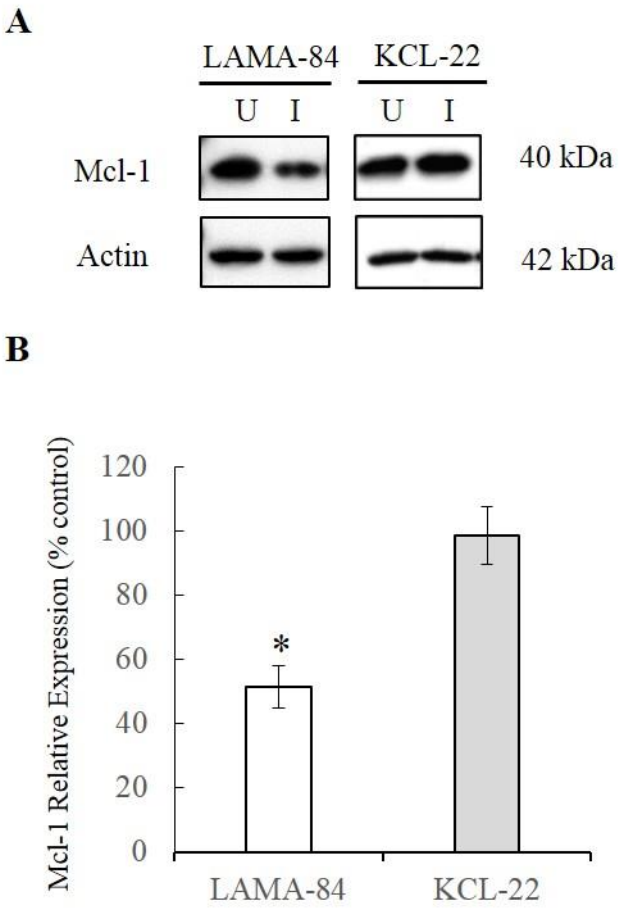

Figure 3.7 Effects of imatinib on expression of Mcl-1 of LAMA-84 cells and KCL-22 cells. Cells were incubated for 24 h in the absence (U) and presence (I) of imatinib (10 μM) and samples were prepared for western blotting for Mcl-1 (40 kDa) and actin (42 kDa). **(A)** shows representative western blots, while **(B)** shows densitometric analysis of the effect of imatinib on Mcl-1 expression. Data expressed as a % of untreated samples which was taken as 100 % (±SEM, n=3) *=p≤0.05 (paired two-tailed student's t-test). Western blot shown is representative from n=3 experiments.

3.3.6 Effects of imatinib and the pan-caspase inhibitor on LAMA-84 and KCL-22 cell apoptosis

The effects of the pan-caspase inhibitor, Z-VAD which blocks activated caspases and hence cell apoptosis, were investigated to confirm that imatinib induces cell death of LAMA-84 cells via induction of apoptosis. The effects of Z-VAD and imatinib on cell viability, cell cycle kinetics and Bcl-2 family protein expressions were therefore determined.

3.3.6.1 Effects of imatinib and the pan-caspase inhibitor, Z-VAD, on cell viability.

After 24 h incubation, imatinib decreased the cell viability of LAMA-84 cells in the absence of Z-VAD. Incubation of cells in the presence of imatinib together with Z-VAD resulted in significantly increased viability (from 61.7 ± 1 % in absence of Z-VAD to 83.1 ± 1.5 % in presence of Z-VAD), ($p \leq 0.05$, n=3) (Figure 3.8). This confirms that imatinib induces caspase-dependent apoptosis in LAMA-84 cells.

While KCL-22 cells only underwent a slight, but statistically insignificant decrease in cell viability after 24 h incubation with imatinib, the presence of Z-VAD did not affect this (from 89.4 ± 0.4 % in the absence of Z-VAD to 88.7 ± 2.9 % in presence of Z-VAD), ($p > 0.05$, n=3) (Figure 3.8). This result is not surprising as imatinib does not induce apoptosis in this cell line.

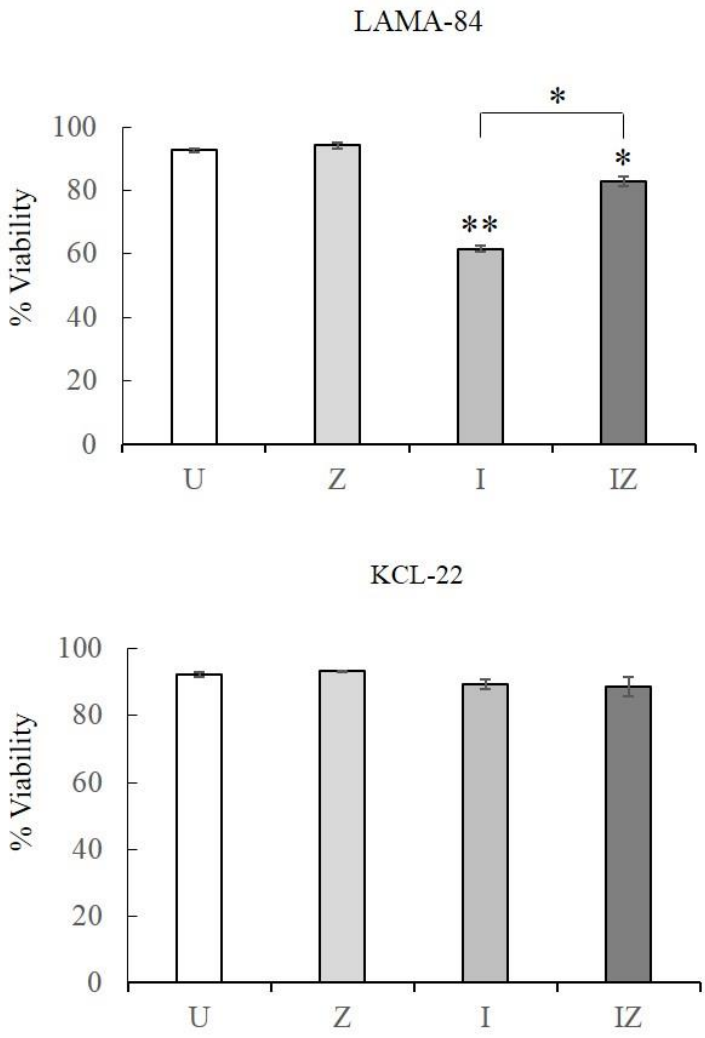

Figure 3.8 Effect of imatinib and the pan-caspase inhibitor, Z-VAD, on LAMA-84 and KCL-22 cell viability. Both LAMA-84 and KCL-22 cells were incubated for 24 h in the absence (U) and presence (I) of imatinib (10 μM), with and without the broad spectrum caspase inhibitor Z-VAD-fmk (Z) (20 μM). Viability was assessed by flow cytometry using the Viacount reagent and protocol (see Materials and Methods). Data are shown as a percentage of viable cells (±SEM, n=3) *=p≤0.05, **= p≤0.01, (paired two-tailed student's t-test).

3.3.6.2 Effects of imatinib and the pan-caspase inhibitor, Z-VAD, on cell cycle kinetics.

After 24 h incubation of LAMA-84 cells in the presence of imatinib there was a large increase in the percentage of cells in the G0 phase of the cell cycle and a corresponding decrease in the number of cells in S-phase and G2-phase (Figure 3.9). The pan-caspase inhibitor, Z-VAD, prevented these changes in cell cycle parameters, confirming induction of apoptosis in LAMA-84 cells by imatinib (from 46.4 ± 1.9 % of cell cycle arrest in G0 in the absence of Z-VAD to 30.5 ± 8.1 % of cell cycle arrest in G0 in the presence of Z-VAD, ($p \leq 0.05$, n=3) (Figure 3.9).

In KCL-22 cells, imatinib induced a partial cell cycle arrest in G1, with fewer cells accumulating in G2. This G1 arrest was particularly evident after 24 h incubation with imatinib, but was not prevented by Z-VAD (from 59.8 ± 4.4 % of cell cycle arrest in G1 in absence of Z-VAD to 56.6 ± 2.9 % of cell cycle arrest in G1 in presence of Z-VAD), ($p > 0.05$, n=3) (Figure 3.9).

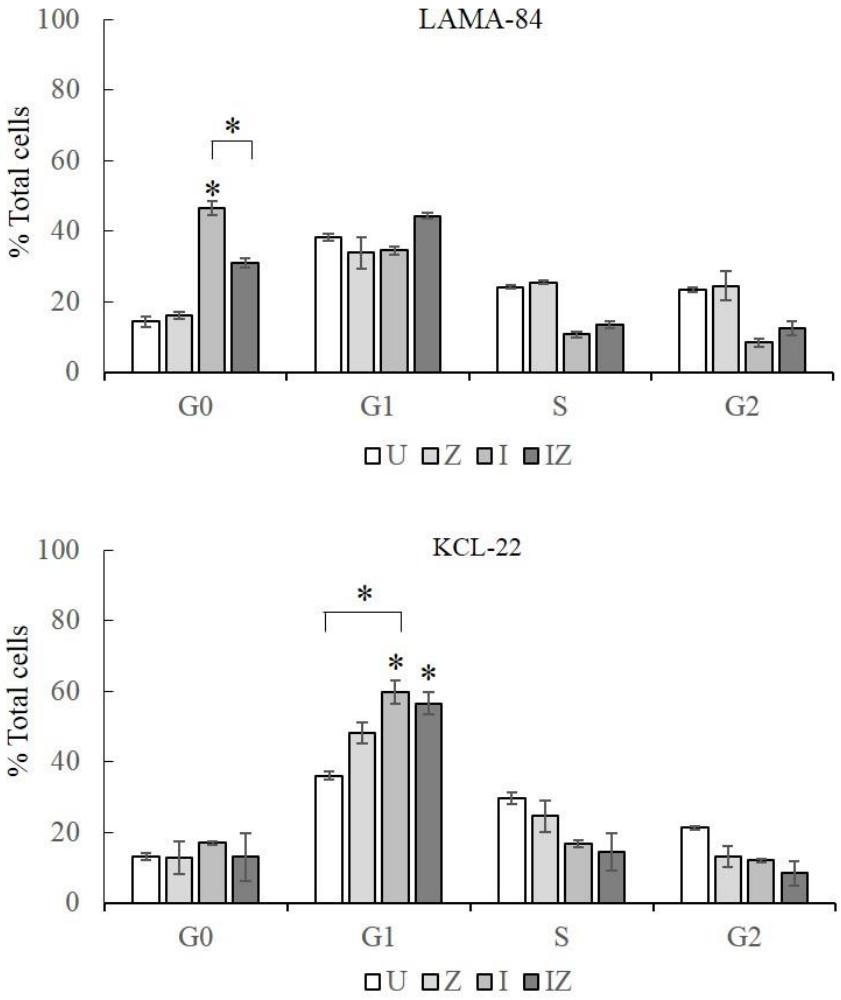

Figure 3.9 Effect of imatinib and the pan-caspase inhibitor, Z-VAD, on LAMA-84 and KCL-22 cell cycle kinetics. Both LAMA-84 and KCL-22 were incubated for 24 h in the absence (U) and presence (I) of imatinib (10 μM), with and without Z-VAD (Z) (20 μM). Cell cycle parameters (expressed as a percentage of the total cell population) were determined by measuring DNA content and flow cytometry (see Materials and Methods). Data are shown as mean (±SEM, n=3) *=$p \leq 0.05$ (paired two-tailed student's t-test).

3.3.6.3 The effects of imatinib and the pan-caspase inhibitor, Z-VAD, on anti-apoptotic protein, Mcl-1 expression.

From previous experiments described earlier in this Chapter, Mcl-1 protein expression was decreased by imatinib in LAMA-84 cells unlike other Bcl-2 family proteins such as Bcl-2, Bcl-X_L, and Bak protein. As a consequence, Mcl-1 expression was investigated further with the pan-caspase inhibitor, Z-VAD. Western blotting was performed to determine the effects of imatinib and Z-VAD on the expression level of this protein in both cell lines.

After 24 h incubation of LAMA-84 cells with imatinib, Mcl-1 levels were significantly decreased in an absence of Z-VAD. In the presence of Z-VAD, the loss of Mcl-1 expression by imatinib was significantly protected (relative expression 58.2 ± 2.8 % from 100 % of control in absence of Z-VAD and 84.3 ± 9.1 % in presence of Z-VAD), ($p \leq 0.05$, n=3) (Figure 3.10A). This confirms that imatinib induced a caspase-mediated decrease in Mcl-1 levels.

In contrast to LAMA-84 cells, imatinib induced only a small and insignificant decrease in Mcl-1 levels in KCL-22 cells in the absence of Z-VAD. In the presence of Z-VAD, this decrease in Mcl-1 was slightly protected (relative expression 98.8 ± 9 % from 100 % of control in absence of Z-VAD to 95.6 ± 9.1 % in presence of Z-VAD), ($p > 0.05$, n=3) (Figure 3.10B). This confirms the caspase-independency of this small decrease in protein level.

To conclude, the decrease in Mcl-1 expression induced by imatinib can be protected by Z-VAD in LAMA-84 cells, but not KCL-22 cells. These results confirm previous experiments in this Chapter that imatinib induces apoptosis in LAMA-84 cells (but not in KCL-22 cells).

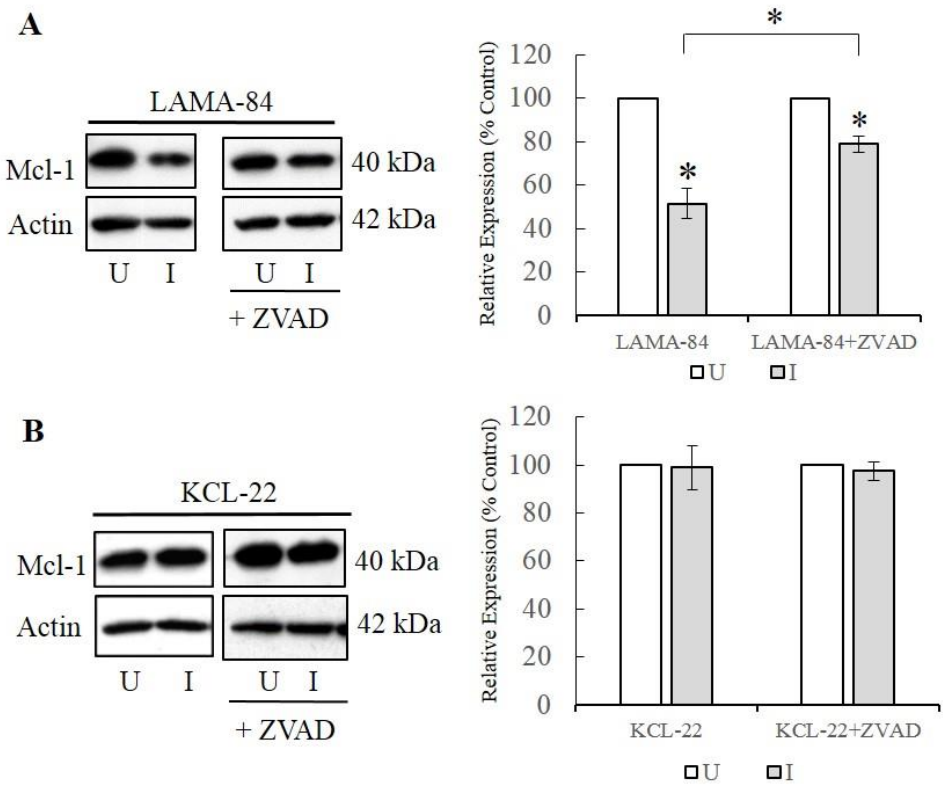

Figure 3.10 Effect of imatinib and the pan caspase inhibitor, Z-VAD, on expression of Mcl-1 in (A) LAMA-84 cells and **(B)** KCL-22 cells. Cells were incubated for 24 h in the absence (U) and presence (I) of imatinib (10 μM) ± ZVAD (20 μM), and samples were prepared for Western blotting for Mcl-1 (40 kDa) and actin (42 kDa), quantified by densitometry. Data expressed as a % of untreated samples (±SEM, n=3) * = $p \leq 0.05$, **= $p \leq 0.01$ (paired two-tailed student's t-test). Western blot shown is representative from n=3 experiments.

3.4 Discussion and Conclusions

The aims of this Chapter were to determine the effects of imatinib on the viability of two CML cell lines, LAMA-84 and KCL-22, in order to establish their usefulness as *in vitro* models to test for the effects of new kinase inhibitors on the growth parameters of TKI-sensitive and -insensitive cell lines. The results in this Chapter confirm previously published results (see section 3.1) and background work in this laboratory, which has shown that LAMA-84 cells are imatinib-sensitive and that KCL-22 cells are relatively imatinib-insensitive. However, new data presented in this Chapter show that imatinib induces cell death in LAMA-84 cells by a caspase-sensitive mechanism, which is likely to be apoptosis, as this drug also induces a rapid (within a few hours) depolarisation of the mitochondrial inner membrane. Unexpectedly, the results also show that whilst imatinib did not induce cell death in KCL-22 cells, it did induce a cell cycle arrest in G1, which was not affected by the presence of the pan-caspase inhibitor.

LAMA-84 cell line was established from the blood of a patient with chronic myeloid leukaemia, in accelerated phase. It is reported to retain the patient's Philadelphia chromosome. However, recent study shows that LAMA-84 cell line has lower BCR-ABL mRNA level compared to another imatinib-resistant cell line, IRK562. Also, it has been reported that LAMA-84 showed greater sensitivity to imatinib compared to IRK562 (Mendonca et al., 2010).

Imatinib significantly decreased LAMA-84 viability at concentrations of 5 μM and above. This finding is in line with several other studies that have used this LAMA-84 cell line as a model imatinib-sensitive CML cell line (Greene et al., 2007, Dasmahapatra et al., 2007, Mendonca et al., 2010). My results show that imatinib induced apoptosis (that was partly reversed by the pan caspase inhibitor) by increasing the number of cells that accumulated in the G0 phase (low DNA content per cell) and induced a decrease in mitochondrial membrane potential (which was also prevented by Z-VAD, data not shown). Caspase-induced activation of apoptosis is usually regulated by changes in function of Bcl-2 family member proteins,

and so the effects of imatinib on the expression levels of several of these proteins was investigated.

For LAMA-84 cells, imatinib treatment did not result in any significant decreases in expression levels of Bcl-2 or Bcl-X_L, two anti-apoptotic members of this family. While it is possible that imatinib affected the localisation or activity of these proteins, no changes in overall levels of these proteins would indicate that they are unlikely to be affected by this drug. However, major changes in the levels of the anti-apoptotic protein, Mcl-1 were observed in LAMA-84 cells after treatment with imatinib, and these decreases were largely prevented by Z-VAD, indicating the importance of caspase activation in this process. A previous report has shown that imatinib decreased the expression of Mcl-1 in K562 cell lines (Aichberger et al., 2005) which, like LAMA-84 cells are imatinib-sensitive CML cell lines (Greene et al., 2007, Deininger et al., 1997). However, this, to my knowledge, is the first report of imatinib-induced decreases in Mcl-1 levels in LAMA-84 cells as an explanation for induction of cell death by this drug.

In contrast, imatinib had no significant effect on the viability of KCL-22 cells. However, surprisingly, it induced a partial cell cycle arrest in G1. This was insensitive to Z-VAD and hence independent of caspase activation, and has not previously been reported. It has been reported that KCL-22 cell line possesses natural resistance to imatinib (Deininger et al., 1997, Mahon et al., 2000). Therefore, this supports the idea that KCL-22 could act as a model for imatinib-insensitive CML for further studies. However, the mechanism of the resistance of KCL-22 to imatinib is still unclear. It has been reported that KCL-22 can resist imatinib without increasing of BCR-ABL expression (Mahon et al., 2000, Ohmine et al., 2003), but other mechanisms might involve BCR-ABL-independent resistance to imatinib (Mahon et al., 2000).

However, a more recent study has shown that KCL-22 have doubled expression of both BCR-ABL mRNA and protein compared to other imatinib-resistant cell lines (Quentmeier et al., 2011). This report also

demonstrated that there was high expression of mutated kinase activity in KCL-22 cells. This point mutation results in an amino acid change in PI3K of E545G. This suggests that this mutation might constitutively activate the PI3K pathway in KCL-22 cells (Quentmeier et al., 2011). Thus, this could partly explain the mechanism of resistance of KCL-22 cell line.

Interestingly, like mature neutrophils (Moulding et al., 2001), KCL-22 cells did not express the anti-apoptotic protein Bcl-2. They did express Bcl-X_L (unlike mature neutrophils) and Mcl-1 (like mature neutrophils), but imatinib did not induce any changes in expression levels of these latter two proteins, in line with its inability to induce apoptosis.

Another important finding from this study was the usefulness of measuring mitochondrial membrane polarisation using the fluorescence indicator JC-1. This dye is cell permeable and "stacks" in the inner membrane of polarised mitochondria (Reers et al., 1995). These JC-1 aggregates in polarised membranes emit red fluorescence (590 nm) when excited at 499 nm. However, upon membrane depolarisation, these aggregates dissociate to the monomeric form of JC-1, which emits green fluorescence (530 nm). This red:green shift can be detected by flow cytometry. An advantage of using this technique to measure apoptosis, is that changes in mitochondrial inner membrane potential are amongst the first cellular changes to be detected during activation of apoptosis. Interestingly, changes in JC-1 fluorescence in LAMA-84 cells were detected within 2-4 h of incubation with imatinib, making it suitable for rapid screening new molecules with the potential to induce apoptosis.

Taken together, the results obtained in this Chapter confirm that these two cell lines can be used as experimental tools to study imatinib-sensitive and imatinib-insensitive CML cells.

CHAPTER 4: EFFECTS OF PURVALANOL A ON IMATINIB-SENSITIVE AND -INSENSITIVE CML CELL LINES

4.1 Introduction

The results in Chapter 3 show that imatinib induces apoptosis in LAMA-84 cells, but has little effect on apoptosis of KCL-22 cells (although it does induce a G1 arrest). Therefore, these two cells lines would appear to be suitable to test the effects of new inhibitors in imatinib-sensitive and –insensitive CML cells.

There is much interest in the development of kinase inhibitors to treat cancers or inflammatory diseases and many new molecules are undergoing clinical trials (Zhang et al., 2009a, Cohen and Alessi, 2013). While new-generation, rationally-designed tyrosine kinase inhibitors are being developed to overcome specific mutations in BCR-ABL, resistance to these new drugs is now emerging (see Introduction). Therefore, there is a need to develop new ways to treat CML cells, perhaps in conjunction with TKIs. Many cancers are resistant to apoptosis or have dysregulated cell cycle control, and so many drugs under development are targeting these two important cellular processes (Elmore, 2007, Hassan et al., 2014).

Cyclin-dependent kinases (CDKs) play important roles in the regulation the cell cycle and deregulation of their activity results in uncontrolled cell proliferation, leading to cancer (McDonald and El-Deiry, 2000). Therefore, several small molecule CDK inhibitors have been developed to block CDK activities. These include the purines (such as olomucine, roscovutine, and purvalanol A) and piperidine-substituted purines (such as flavopiridol, indirubin, and paullones) (reviewed in (Knockaert et al., 2002a)).

Although these inhibitors have diverse chemical structures, they all bind to the ATP-binding site of CDKs. These inhibitors generate their anti-proliferative effects by arresting the cell cycle in G1 and/or G2/M phase (Knockaert et al., 2002a). Various studies have evalauted the effects of these inhibitors on cancer cells (Damiens and Meijer, 2000, Senderowicz and Sausville, 2000) and some of these inhibiters have shown promising results in many types of cancer, including CML (Fischer and Lane, 2000, Bright et al., 2010, Fisher and Morgan, 1994).

One of the most potent CDK inhibitors developed to date is purvalanol A, which binds between the 2, 6, 9-tri substituted purines and the ATP-binding site of human CDK2 (Gray et al., 1998, Gray et al., 1999), resulting in competitive inhibition of ATP-binding. This inhibition induces cell cycle arrest and cell apoptosis in both mouse fibroblasts and in several cancer cell lines (Villerbu et al., 2002). In addition, recent studies in human cancer cell lines showed that purvalanol A treatment inhibits both CDK activity and the expression of several anti-apoptotic proteins of the Bcl-2 family, such as Bcl-2 and Bcl-X_L (Iizuka et al., 2007, Iizuka et al., 2008).

Purvalanol A was found to interact with purified CDK2 and this may explain its anti-mitotic properties (Knockaert et al., 2002a). However, its actual intracellular targets are still undefined. One group reported that purvalanol also interacts with p42/p44 MAPK protein in various cell lines, as detected by affinity chromatography (Knockaert et al., 2002b). In addition, purvalanol inhibited p42/p44 MAPK activities in a dose-dependent manner in the Chinese hamster lung fibroblast cell line, CCL39 (Knockaert et al., 2002b). This suggests that the anti-proliferative effects of purvalanol may be mediated by both CDK inhibition and interactions with p42/p44 MAPK.

Purvalanol A was identified in a previous project in our laboratory that screened a Tocris Kinase Inhibitor array for effects on the viability of LAMA-84 and KCL-22 cells, in the presence and absence of imatinib. Purvalanol A showed promising results by significantly decreasing cell viability of KCL-22 cells (Guest, 2012). Thus, the effects of purvalanol A

on the molecular signalling pathways regulating apoptosis in the imatinib-sensitive and imatinib-insensitive CML cell lines are the major focus of this Chapter.

The aims of the work described in this Chapter were:

i) To determine the effects of purvalanol A on the growth and apoptosis of these two cell lines.

ii) To measure the expression of Bcl-2 family members in these two cell lines and to determine how their protein levels are affected by purvalanol A.

iii) To investigate the effects of these two inhibitors on the activation status of intracellular signalling systems that are directly or indirectly regulated by BCR-ABL activity.

4.2 Methods

All methods used were as described in Chapter 2. Purvalanol A was dissolved in DMSO, and so control suspensions contained the same volume of DMSO as was used for purvalanol A, and these had no effect on any of the parameters measured (result not shown).

4.3 Results

Initial experiments measured the effects of purvalanol A on apoptosis of these cell lines using the Viacount assay, cell number, cell morphology and cell cycle kinetics. Also, changes in the mitochondrial membrane potential were measured as the red:green shift in fluorescence of JC-1. The levels of expression of pro- and anti-apoptotic proteins, such as Mcl-1, Bcl-2, Bcl-X_L, and Bak, plus the effects of the pan-caspase inhibitor Z-VAD were also measured. The effects of purvalanol A on the activation status of several intracellular signalling systems, including Erk, STAT3, p38 and Akt were also investigated. Lastly, the effects of purvalanol A with BIRB796, p38 MAPK inhibitor, were also measured.

4.3.1 Effects of purvalanol A on the viability of LAMA-84 and KCL-22 cell lines

LAMA-84 and KCL-22 cell lines were initially tested for their sensitivity to purvalanol A, in terms of the cell viability. LAMA-84 and KCL-22 cells were incubated for 24 h in the presence and absence of purvalanol A at different concentrations, and viability was assessed using the Viacount assay.

Purvalanol A induced a dose-dependent decrease in cell viability of both cell lines (Figure 4.1). However, purvanalol A had a greater effect on the viability of KCL-22 cells than on LAMA-84 cells at each dose tested and these differences were highly significant ($P \leq 0.001$, two-way ANOVA with replication). The maximal effect of purvalanol A on both cell lines was achieved at a concentration of 30 μM. Thus, this concentration was used in further experiments unless stated otherwise.

When used at a concentration of 30 μM, purvalanol A significantly induced apoptosis in LAMA-84 cell line, ($p \leq 0.01$, $n=3$). After 24 h of incubation with purvalanol A, LAMA-84 cell viability was decreased significantly (from 92.7 ± 0.5 % cell viability of control samples to 63.8 ± 0.6 % cell viability of purvalanol A treated samples). Similarly, purvalanol A significantly decreased cell viability in KCL-22 cells line which are insensitive to imatinib ($p \leq 0.01$, $n=3$). KCL-22 cell viability was decreased from 92.4 ± 0.6 % of control samples to 30 ± 3.2 % in samples treated for 24 h with 30 μM purvalanol A (Fig. 4.1).

The effects of purvalanol A in combination with imatinib on viability of these cell lines were then measured. Both cell lines were incubated for 24 h with the combination of both 10 μM imatinib and 30 μM purvalanol A. However, there was no additive inhibitory effect observed when imatinib and purvalanol were used in combination, ($p > 0.05$, $n=3$) (Figure 4.2).

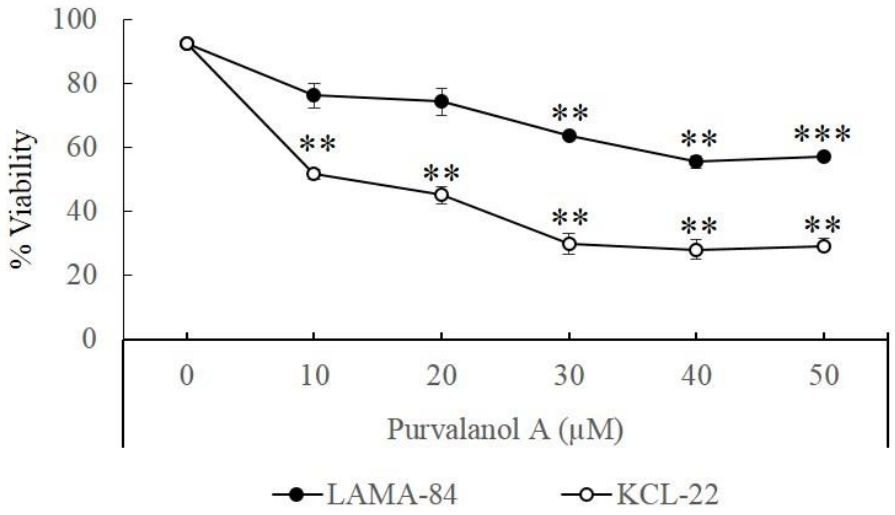

Figure 4.1 Differential sensitivity of LAMA-84 and KCL-22 cell lines to purvalanol A. LAMA-84 and KCL-22 cell lines were incubated in the absence and presence of gradient concentrations of purvalanol A (10, 20, 30, 40, and 50 µM). Viability was determined using the Viacount assay, in control cells, or cells incubated with gradient concentrations of purvanol A (P), as follows: LAMA-84 cell line ●; KCL-22 cell line ○. Data are shown as mean (±SEM, n=3) **=p≤0.01, ***=p≤0.001 (paired two-tailed student's t-test).

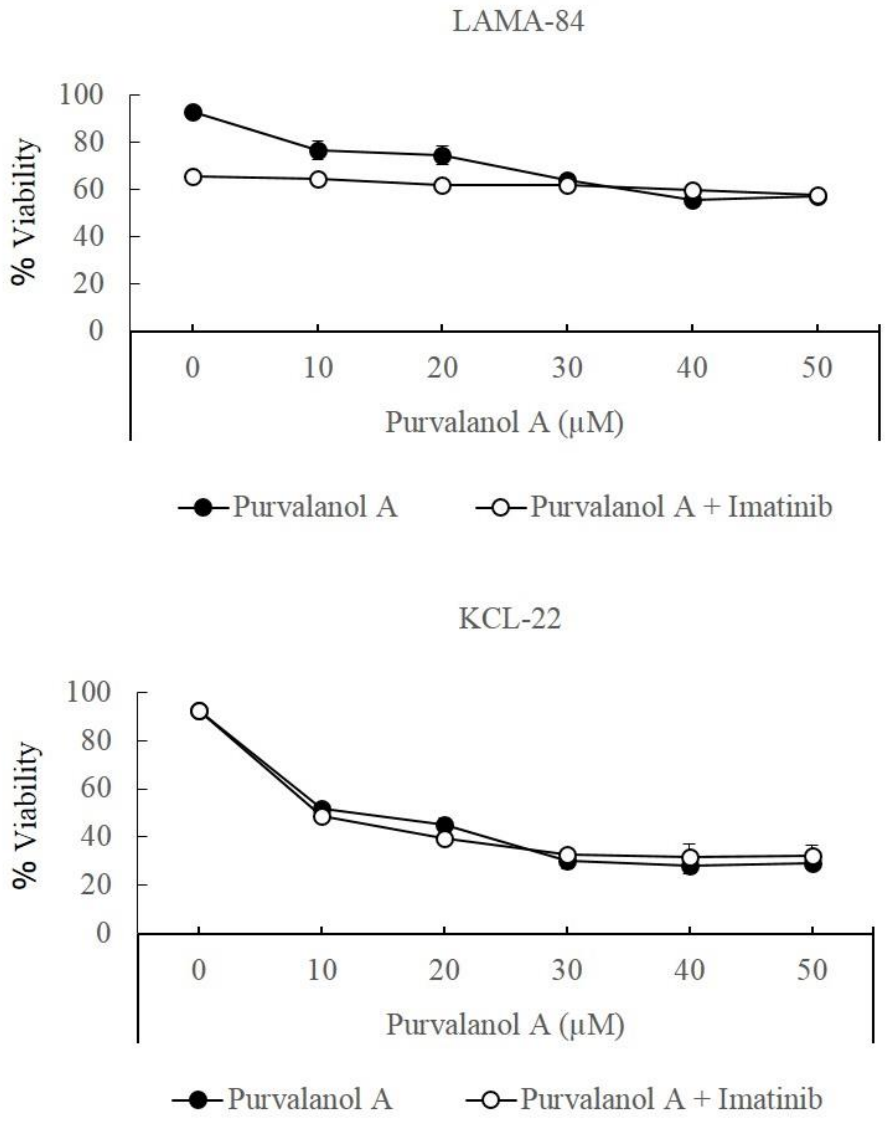

Figure 4.2 Differential sensitivity of LAMA-84 and KCL-22 cell lines to imatinib and purvalanol A. LAMA-84 and KCL-22 cells were incubated with gradient concentrations of purvalanol A together with a constant concentration of imatinib (10 μM). Values show are % of cell viability, as follows: purvalanol A treated ●; imatinib plus purvalanol A treated ○. Data are shown as mean (±SEM, n=3).

4.3.2 Effects of purvalanol A on cell number and morphology of LAMA-84 and KCL-22 cell lines

The number of cells and cell morphology were assessed by flow cytometry and light microscopy, respectively. Both LAMA-84 and KCL-22 cells were incubated for 24 h in the absence (untreated control) and presence of 10 μM imatinib and 30 μM purvalanol A and then were counted and viewed microscopically to confirm the levels of apoptosis.

Cell counts by flow cytometry (Figure 4.3) showed that imatinib significantly decreased the total cell count of LAMA-84 cells, but not KCL-22 cells, confirming previous experiments (Chapter 3). In contrast to the effects of imatinib, purvalanol A decreased the total number of cells counted in both cell lines. In LAMA-84 cells, the total number of cells counted was decreased from $2.2 \times 10^6 \pm 1.7 \times 10^5$ untreated cells/mL to $1.3 \times 10^6 \pm 1.4 \times 10^4$ cells/mL after 24 h incubation, ($p \leq 0.05$, n=3). Similarly, purvalanol A decreased the total number of KCL-22 cells counted from $2.2 \times 10^6 \pm 1.3 \times 10^5$ untreated cells/mL to $1.4 \times 10^6 \pm 4.8 \times 10^4$ cells/mL after 24 h incubation, ($p \leq 0.05$, n=3).

In addition, the combination treatment of imatinib and purvalanol decreased the total cell counts significantly, ($p \leq 0.05$, n=3, respectively) in both cell lines ($1.3 \times 10^6 \pm 6.5 \times 10^4$ cells/mL and $1.3 \times 10^6 \pm 1.1 \times 10^5$ cells/mL in LAMA-84 and KCL-22 cells, respectively). However, the combined effects of these two drugs on cell numbers were not significantly different to those obtained by the use of purvalanol A alone, ($p > 0.05$, n=3). These results correlate with the viability changes observed when the cells were treated with imatinib and purvalanol A using the Viacount assay.

Analysis of cell morphology showed that purvalanol A induced apoptosis in both LAMA-84 and KCL-22 cells, as detected by appearance of apoptotic cells with condensed chromatin (Figure 4.4).

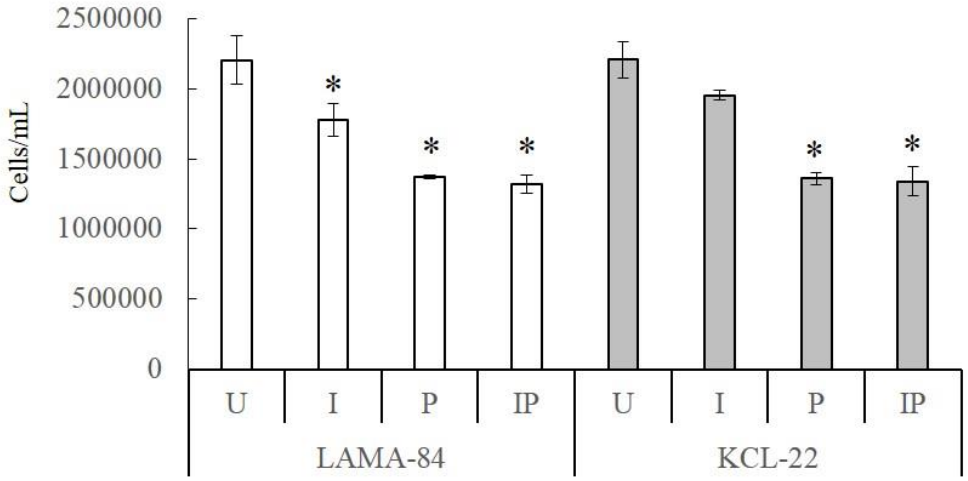

Figure 4.3 Effects of imatinib and purvalanol A on the number of LAMA-84 and KCL-22 cells. Both LAMA-84 and KCL-22 cells were incubated for 24 h in the absence (U) and presence (I) of imatinib (10 μM), (P) purvalanol A (30 μM), and (IP) imatinib (10 μM) with Purvalanol A (30 μM). Total cell counts were assessed by flow cytometry using the Viacount reagent and protocol (see Materials and Methods). Data are shown as mean (±SEM, n=3) *= $p \leq 0.05$ (paired two-tailed student's t-test).

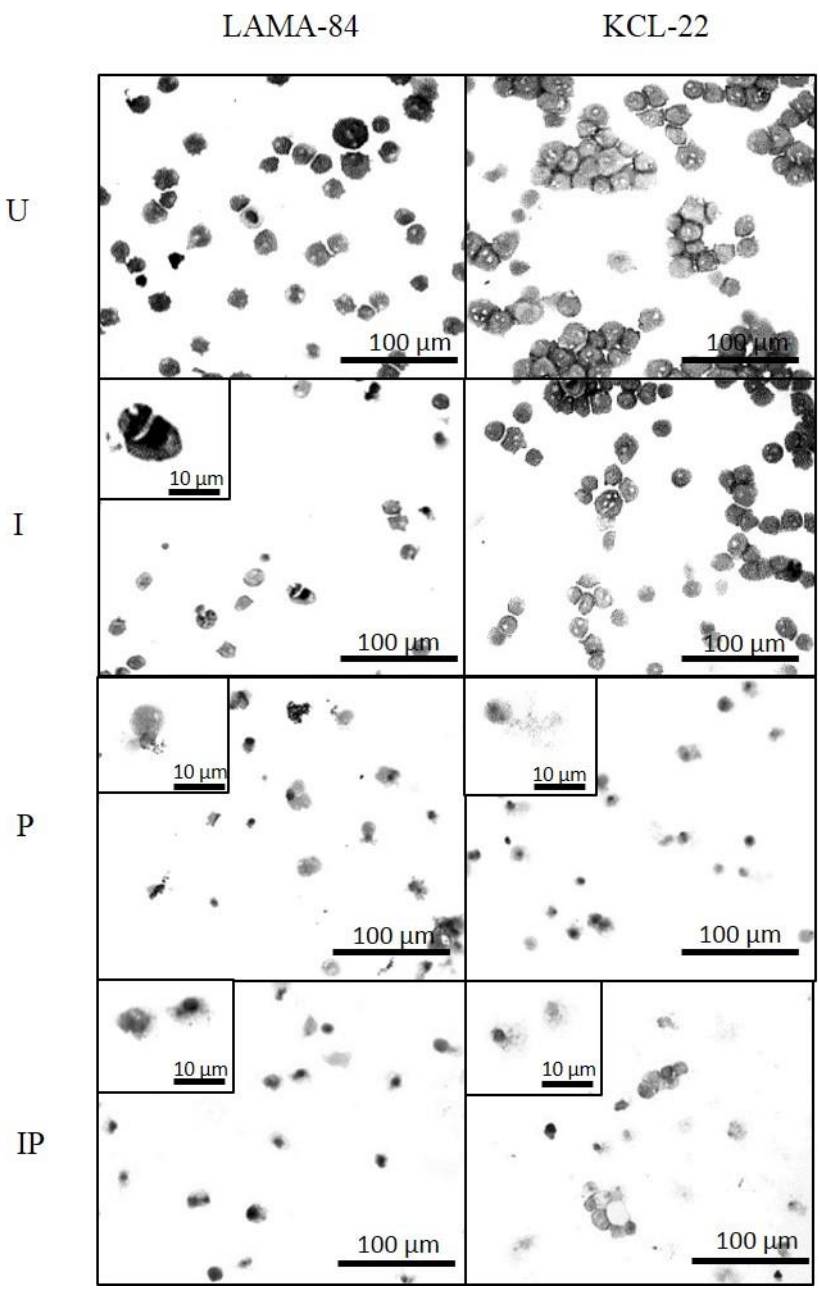

Figure 4.4 Effect of imatinib and purvalanol A on LAMA-84 and KCL-22 cell morphology. Both LAMA-84 and KCL-22 cells were incubated for 24 h in the absence (U) and presence (I) of imatinib (10 µM), (P) purvalanol A (30 µM), and (IP) with the combination of imatinib (10 µM) and purvalanol A (30 µM). Cell morphology was analysed by light microscope (see Materials and Methods). Inset shows cells viewed at higher magnification.

4.3.3 Effects of purvalanol A on cell cycle kinetics of LAMA-84 and KCL-22 cell lines

Results in Chapter 3 showed that imatinib resulted in a significant increase of G0 cells in the population of LAMA-84 cells, but not in KCL-22 cells. In this Chapter, the effects of purvalanol A on cell cycle kinetics were measured in order to compare the effects with those of imatinib.

LAMA-84 cells and KCL-22 cells were incubated for 24 h in the presence and absence of gradient concentrations of purvalanol A (10, 20, 30, 40, and 50 µM) prior to measuring cell cycle kinetics. As shown in Figure 4.5, 10-30 µM purvalanol A treatment caused a significant increase in the number of cells in the G0 phase in both cell lines and 30 µM showed maximal effect, ($p \leq 0.05$, n=3) (Figure 4.5A).

30 µM purvalanol A significantly induced cell cycle accumulation in G0 in LAMA-84 cells from 14.4 ± 1.5 % of the total population of untreated samples to 40.6 ± 3.4 % of total in population purvalanol A treated samples after 24 h incubation. In KCL-22 cells, purvalanol A induced a significant increase in G0 (45.6 ± 1.5 % of the total cell population in purvalanol A treated samples compared to 13.1 ± 0.8 % of the total cell population in untreated samples), with fewer cells accumulating in G2 (data not shown). In contrast to imatinib, purvalanol A did not cause cell accumulation in G1 in either of the cell lines (Figure 4.5 B)

However, the combination treatment did not show any increased accumulation in the G0 population compared to purvalanol A alone, ($p > 0.05$, n=3) (Figure 4.6).

To conclude, the results of this cell cycle analysis appeared to confirm those of the Viacount assay, in that purvalanol A, used alone, induces apoptosis in both imatinib-sensitive and –insensitive CML cell lines.

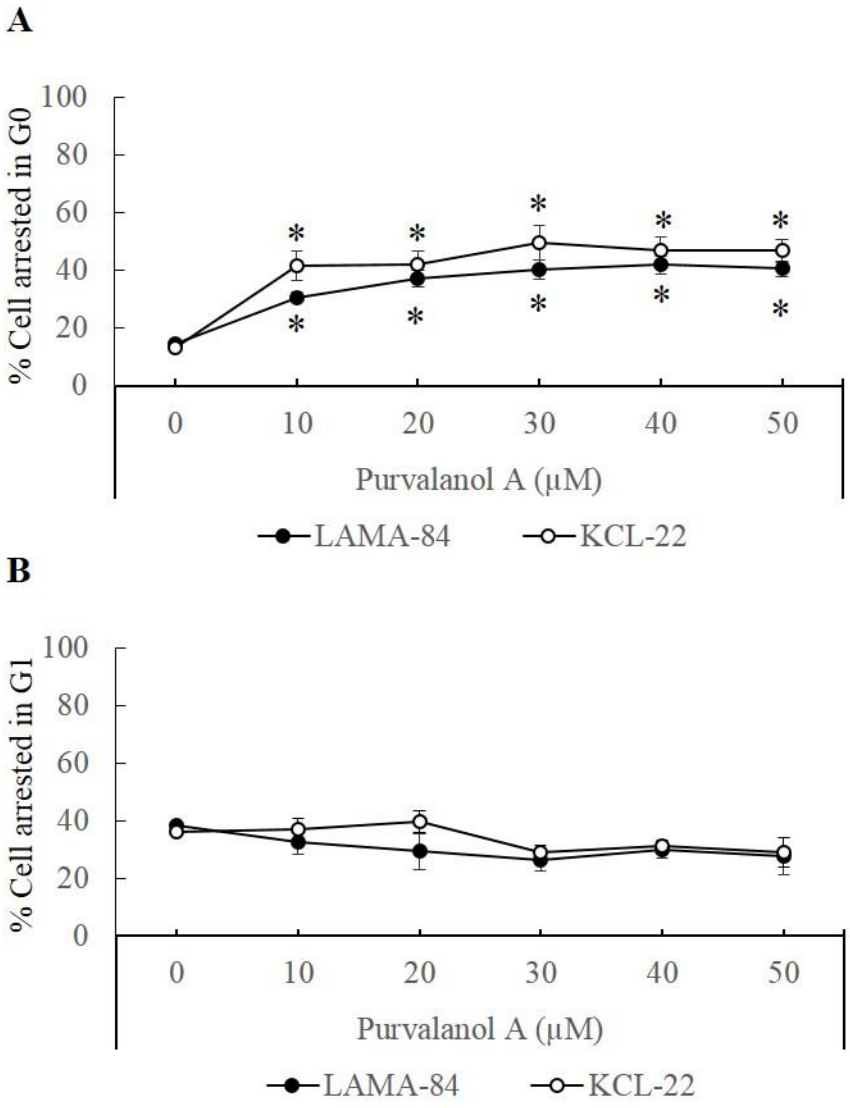

Figure 4.5 Effect of purvalanol A on LAMA-84 and KCL-22 cell cycle kinetics. Cell cycle parameters (expressed as a percentage of the total cell population) of cell arrested in G0 **(A)** and G1 **(B)** were determined by measuring DNA content and flow cytometry. Data are shown as mean (±SEM, n=3) *=p≤0.05 (paired two-tailed student's t-test).

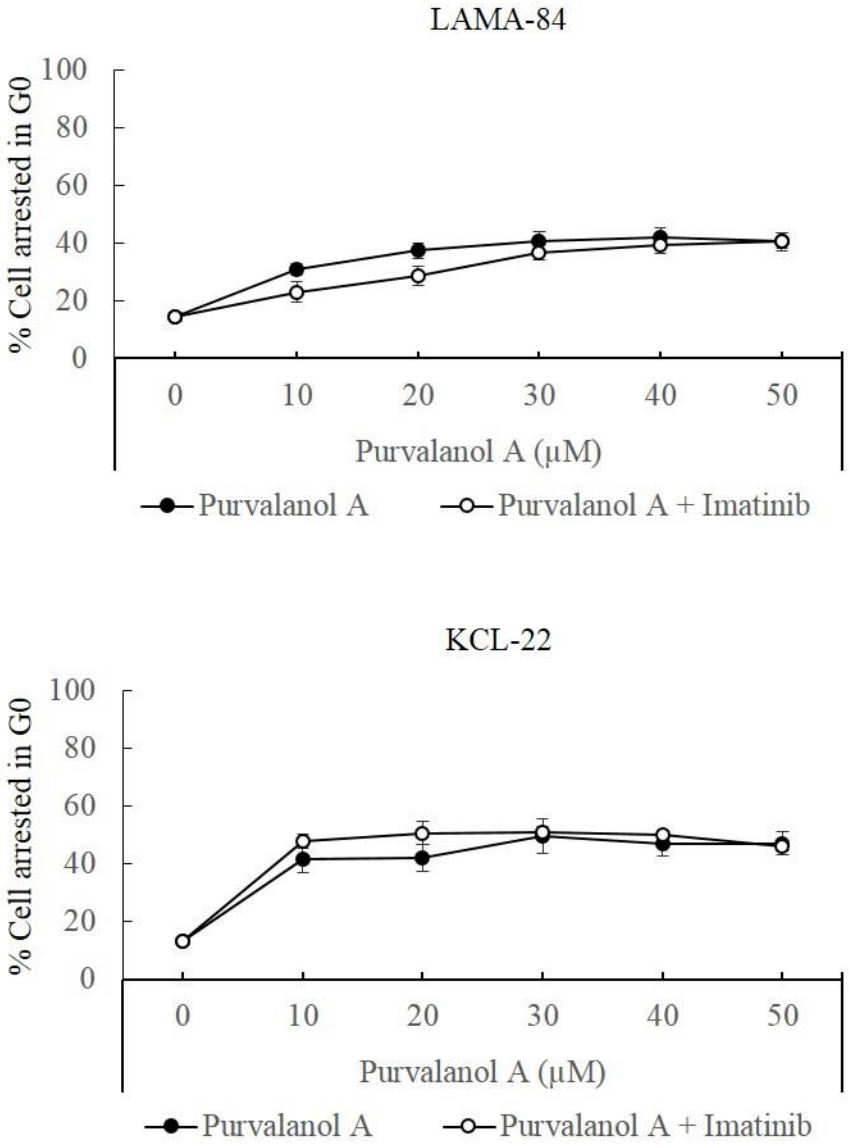

Figure 4.6 Effects of purvalanol A alone and with imatinib on LAMA-84 and KCL-22 cell cycle kinetics. LAMA-84 and KCL-22 cells were incubated with gradient concentrations of purvalanol A together with a constant concentration of imatinib (10 µM). Values show are % of cells arrested in G0, as follows: purvalanol A treated ●; imatinib plus purvalanol A treated ○. Data are shown as mean (±SEM, n=3).

4.3.4 Effects of purvalanol A on mitochondrial membrane potential of LAMA-84 and KCL-22 cell lines.

Changes in mitochondrial inner membrane potential can be detected by the JC-1 assay (see Chapter 3). This assay was performed in order to measure the initial events controlling apoptosis induced by purvalanol A. Both cell lines were treated with imatinib, purvalanol A, and imatinib and purvalanol A together for 0 h, 30min, 1, 2, 4, 6, 18, 24 h. After incubation, all samples were then incubated with JC-1 before measuring red and green fluorescence by flow cytometry.

Figure 4.7 shows that purvalanol A induced mitochondrial membrane depolarisation in both LAMA-84 and KCL-22 cells. In LAMA-84 cells, purvalanol A caused a significant mitochondrial depolarisation change that was detected within 2 h of incubation (4.9 ± 0.5 % at 0 h to 15.6 ± 0.4 % after 2 h purvalanol A incubation), ($p \leq 0.05$, n=3).

In KCL-22 cells, purvalanol A also significantly induced mitochondrial polarisation within 2 h of incubation ($p \leq 0.05$, n=3), whereas imatinib had no effect on this cell line. Purvalanol A induced mitochondrial depolarisation from 8.7 ± 0.3 % at 0 h to 17.6 ± 0.8 % after 2 h purvalanol A incubation (Figure 4.7). This indicates that purvalanol A caused a rapid change in mitochondrial function associated with apoptosis in both cell lines. There was, however, no additive effect of purvalanol A and imatinib when used together, in either cell line.

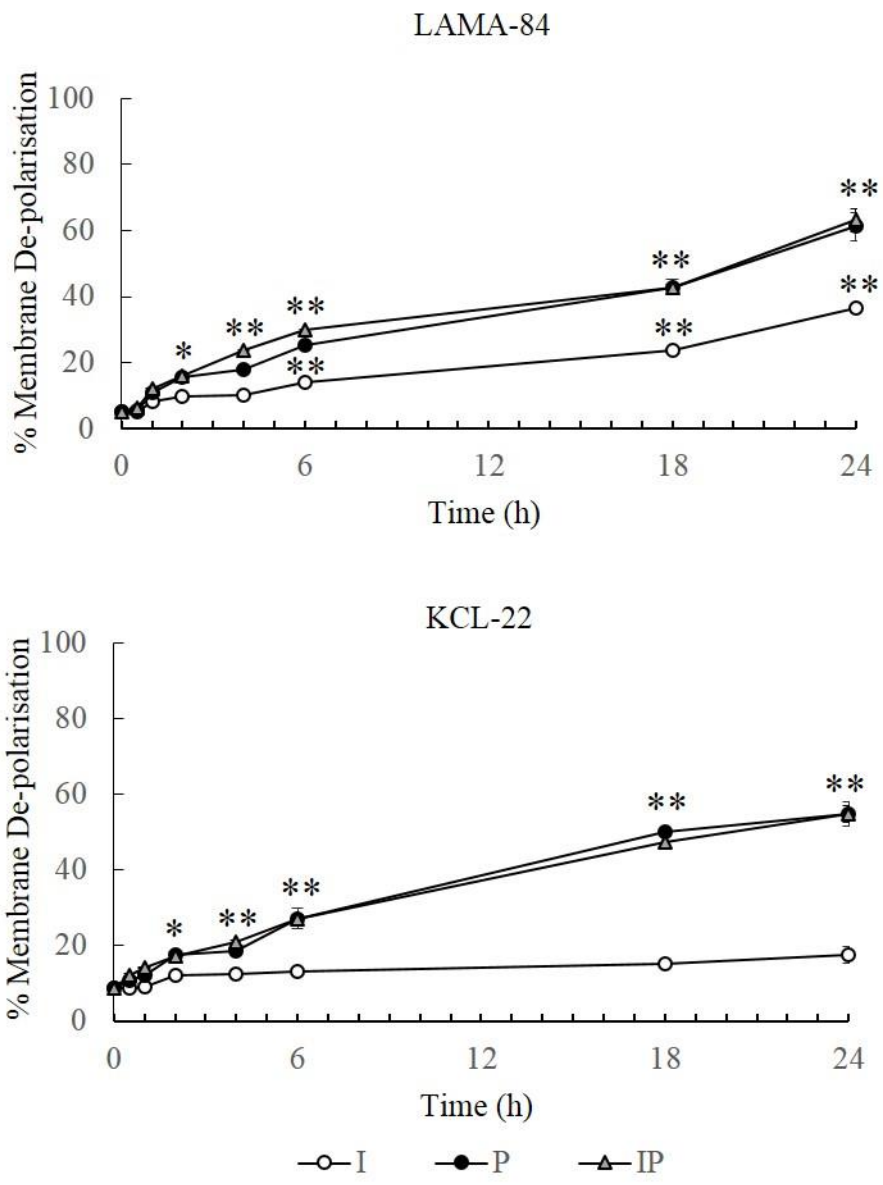

Figure 4.7 Effects of Imatinib and Purvalanol A on mitochondrial membrane potential in LAMA-84 and KCL-22 cells. Both LAMA-84 and KCL-22 cell lines were incubated with imatinib (I, 10 μM) or purvalanol A (P, 30 μM) or the combination of imatinib and purvalanol A (IP) (Δ). Values shown are % of cells showing mitochondrial membrane depolarization (no depolarization was detected in control cells incubated under identical conditions). Data are shown as mean (±SEM, n=3) * = $p \leq 0.05$, **=$p \leq 0.01$ compared to 0 h timepoint (One-way ANOVA followed by *Bonferroni post hoc* comparisons tests).

4.3.5 Effects of purvalanol A on expression of the Bcl-2 family proteins in LAMA-84 and KCL-22 cell lines

Results in Chapter 3 showed that imatinib had no significant effect on the expression of members of the Bcl-2 family, except for decreased Mcl-1 expression in LAMA-84 cells. Thus, the effects of purvalanol A on the expression levels of Bcl-2 family proteins in both LAMA-84 and KCL-22 cell lines were measured by western blotting. Both cell lines were treated with purvalanol A for 24 h and protein extracts prepared. Western blots were then performed using a range of anti-human Bcl-2 family antibodies to detect expression of Bcl-2, Bcl-X_L, Bak, and Mcl-1.

4.3.5.1 Anti-apoptotic protein, Bcl-2

In LAMA-84 cells, purvalanol A resulted in a slight decrease in Bcl-2 expression, but this decrease did not reach statistical significance, ($p>0.05$, $n=3$) when compared to untreated samples. In contrast, KCL-22 cells did not express Bcl-2 protein (Figure 4.8) and purvalanol A did not induce its expression.

4.3.5.2 Anti-apoptotic protein, Bcl-X_L

Purvalanol A resulted in a slight decrease in Bcl-X_L expression in both LAMA-84 and KCL-22 cells, but while this decrease was greater in KCL-22 compared to LAMA-84 cell line, these change in expression did not reach statistical significance when compared to control, untreated cells, ($p>0.05$, $n=3$) (Figure 4.8).

4.3.5.3 Pro-apoptotic protein, Bak

Similar to imatinib, purvalanol A resulted a slight increase in expression of the pro-apoptotic Bak protein in both cell lines, but these changes did not reach statistical significance ($p>0.05$, $n=3$) (Figure 4.8).

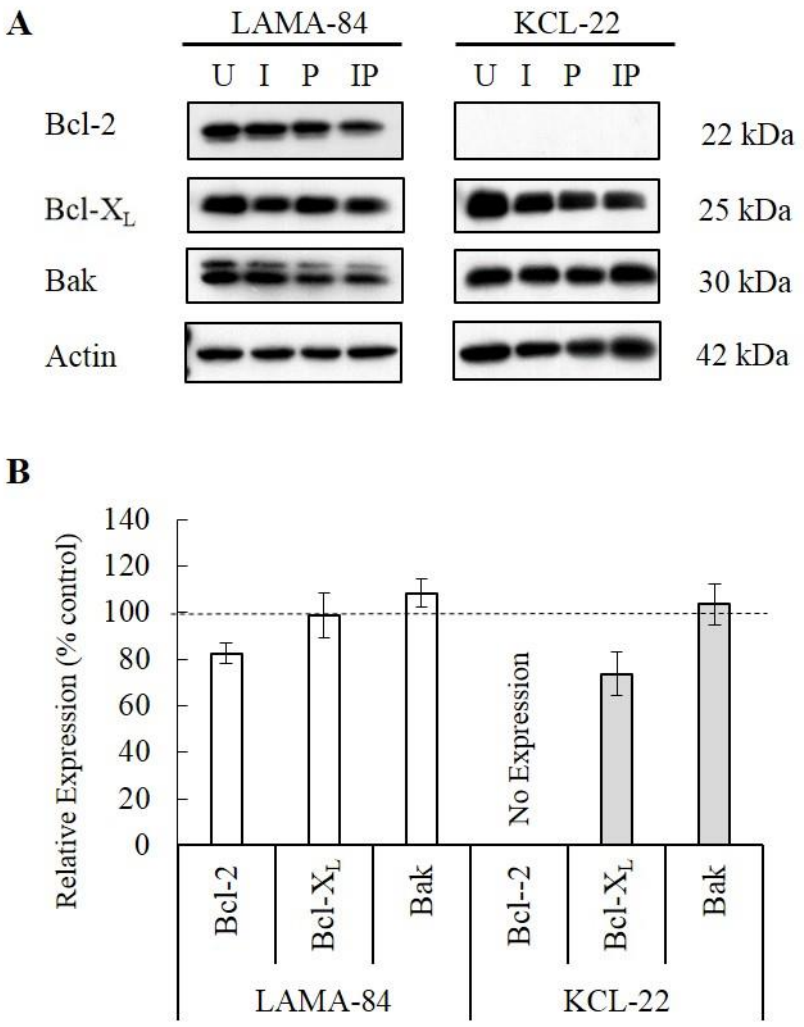

Figure 4.8 Effects of Purvalanol A and imatinib on expression of Bcl-2, Bcl-X_L, and Bak in LAMA-84 cells and KCL-22 cells. (A) Cells were incubated for 24 h in the absence (U) and presence of (I) imatinib, (P) purvalanol A, and (IP) imatinib with purvalanol A and samples were prepared for Western blotting for Bcl-2 (22 kDa), Bcl-X_L (25 kDa), Bak (30 kDa) and actin (42 kDa). **(B)** Densitometric analysis of the effects of purvalanol A on Bcl-2, Bcl-X_L, and Bak expression. Data are expressed as a % of untreated samples (±SEM, n=3). Western blot shown is representative from n=3 experiments.

4.3.5.4 Anti-apoptotic protein, Mcl-1

Results in Chapter 3 showed that imatinib significantly decreased the levels of Mcl-1 expression in LAMA-84 cells, but had no effect in KCL-22 cells.

In contrast to the effects of imatinib, purvalanol had very little effect on Mcl-1 expression in LAMA-84 cells. 24 h incubation with purvalanol A resulted in a decrease in Mcl-1 expression, but this decrease did not reach statistical significance (relative expression of 69.2 ± 6.9 % compared to 100 % of control), ($p>0.05$, $n=3$). However, purvalanol A resulted in a significant decrease in Mcl-1 expression in KCL-22 cells. Mcl-1 expression was barely detectable after 24 h incubation with purvalanol A (relative expression 2.6 ± 1.8 % compared to 100 % of control), ($p\leq0.01$, $n=3$) (Figure 4.9).

To conclude, purvalanol A, like imatinib, caused no significant changes in expression of Bcl-2, Bcl-X_L, and Bak proteins. However, the effects of purvalanol A on Mcl-1 expression were totally different in both cell lines. In contrast to imatinib, purvalanol A caused a significant decrease in expression of anti-apoptotic protein, Mcl-1 in KCL-22 cells, but did not significantly alter expression of this protein in LAMA-84 cells.

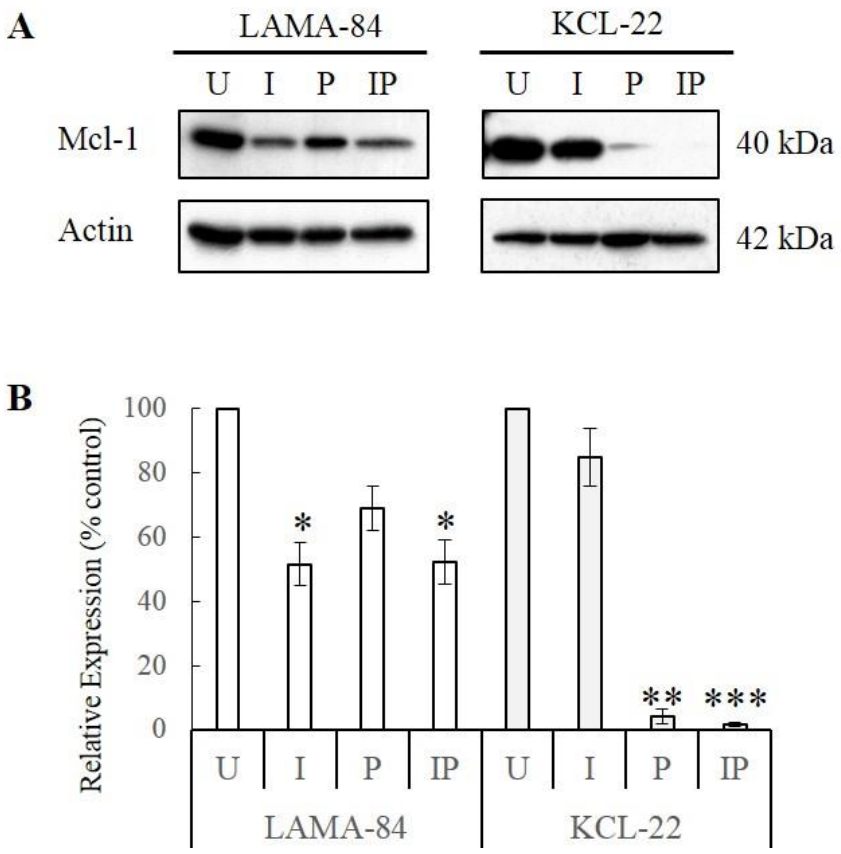

Figure 4.9 Effects of imatinib and purvalanol A on expression of Mcl-1 in LAMA-84 cells and KCL-22 cells. (A) Cells were incubated for 24 h in the absence (U) and presence (I) of imatinib (10 μM) or (P) purvalanol A (30 μM) and samples were prepared for Western blotting for Mcl-1 (40 kDa) and actin (42 kDa). Representative blots are shown in (A). **(B)** Densitometric analysis of the effects of purvalanol A on Mcl-1 expression. Data are shown as mean (±SEM, n=3) *=p≤0.05, **=p≤0.01, ***=p≤0.001 (paired two-tailed student's t-test). Western blot shown is representative from n=3 experiments.

4.3.6 Effects of purvalanol A and the pan-caspase inhibitor on LAMA-84 and KCL-22 cell apoptosis

The effects of the pan-caspase inhibitor, Z-VAD, were measured to evaluate the role of caspases (and hence apoptosis) on the effects of purvalanol A on CML cells.

4.3.6.1 Effects of Purvalanol A and the pan-caspase inhibitor, Z-VAD, on cell viability.

In LAMA-84 cell lines, after 24 h incubation with purvalanol A cell viability was significantly decreased in the absence of Z-VAD. However, in the presence of the Z-VAD cell viability of purvalanol A treated LAMA-84 cells was unaffected (63.8 ± 0.6 % cell viability in absence of Z-VAD and 66.3 ± 7.8 % cell viability in presence of Z-VAD) ($p>0.05$, $n=3$) (Figure 4.10). This is different from the effect of imatinib on cell viability that induced caspase-dependent apoptosis in LAMA-84 cells.

In KCL-22 cells, 24 h incubation with purvalanol A decreased cell viability significantly in the absence of Z-VAD but viability was also largely unaffected by the presence of Z-VAD (Figure 4.10). This result also indicates a low level of caspase-dependent decrease in cell viability triggered by purvalanol A.

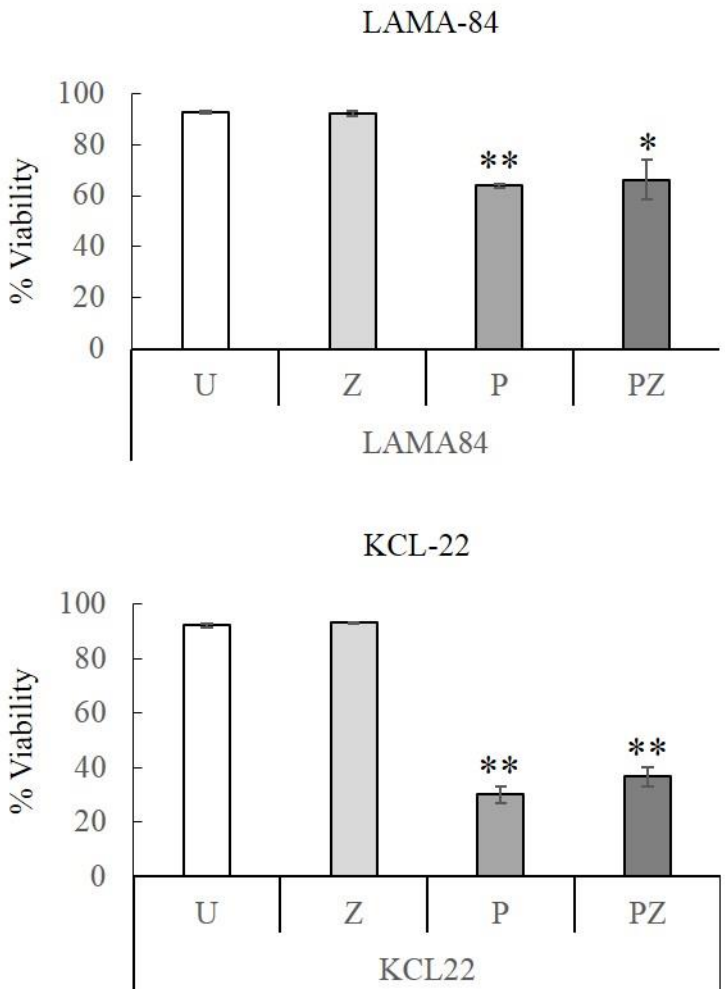

Figure 4.10 Effects of purvalanol A and the pan caspase inhibitor, Z-VAD, on CML cell viability. Both LAMA-84 and KCL-22 cell lines were incubated for 24 h in the absence (U) and presence (P) of purvalanol A (30 μM), with and without the broad spectrum caspase inhibitor Z-VAD-fmk (Z) (20 μM). Viability was assessed by flow cytometry using the Viacount reagent and protocol (see Materials and Methods). Data are shown as a percentage of viable cells (±SEM, n=3) *=p≤0.05, **=p≤0.01 (paired two-tailed student's t-test).

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4.3.6.2 Effects of purvalanol A and the pan-caspase inhibitor, Z-VAD, on cell cycle kinetics.

In LAMA-84 cells, after 24 h incubation of purvalanol A, there was a large increase in the percentage of cell population in the G0 phase of the cell cycle in absence of Z-VAD. The presence of Z-VAD did not prevent this cell cycle accumulation in G0 (Figure 4.11). This is similar to results obtained by Viacount assay (Section 4.3.6.1).

In KCL-22 cells, the caspase-independent apoptosis caused by purvalanol A was confirmed by cell cycle analysis. Purvalanol A caused a large increase in cell cycle accumulation in G0 in both the absence and presence of Z-VAD, but these differences were not statistically significant, ($p>0.05$, $n=3$), (45.6 ± 1.5 % of cell cycle accumulation in G0 in the absence of Z-VAD and 55 ± 1.8 % of cell cycle accumulation in G0 in the presence of Z-VAD) (Figure 4.11). This confirms that this phenomenon was caspase-independent.

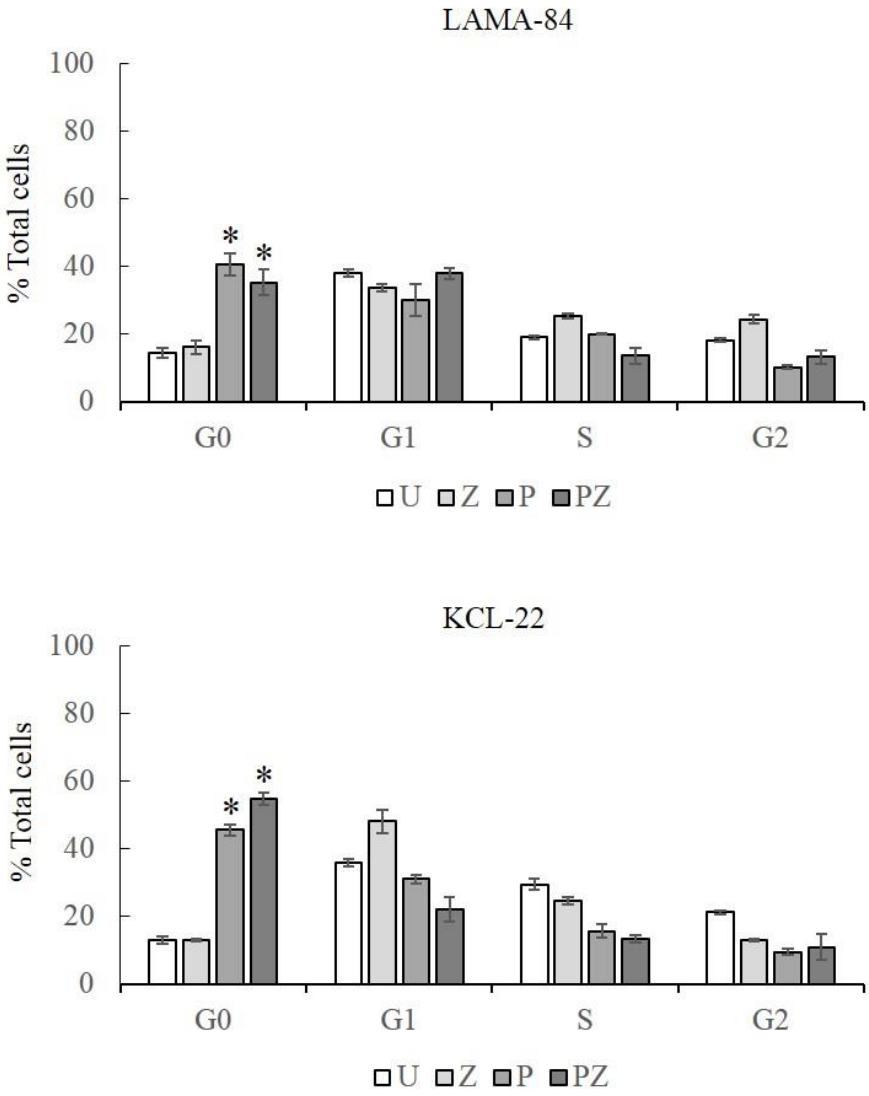

Figure 4.11 Effects of purvalanol A and the pan caspase inhibitor (Z-VAD-fmk) on CML cell cycle kinetics. Both LAMA-84 and KCL-22 cell lines were incubated for 24 h in the absence (U) and presence (P) of purvalanol A (30 μM), with and without Z-VAD (Z) (20 μM). Cell cycle parameters (expressed as a percentage of the total cell population) were determined by measuring DNA content and flow cytometry (see Materials and Methods). Data are shown as mean (±SEM, n=3) *=$p \leq 0.05$ (paired two-tailed student's t-test) compared to untreated control (U).

4.3.6.3 Effects of purvalanol A and the pan-caspase inhibitor, Z-VAD-fmk on expression of the anti-apoptotic protein, Mcl-1 expression.

Western blotting was performed to determine the effects of purvalanol A together with the pan-caspase inhibitor on the expression of Mcl-1 in both cell lines. After 24 h incubation of LAMA-84 cells with purvalanol A, Mcl-1 levels decreased in absence of Z-VAD. However, in the presence of Z-VAD, the Mcl-1 levels were approximately the same compared to levels observed in the absence of Z-VAD. (88.7 ± 3.5 % in absence of Z-VAD to 86.5 ± 8.7 % in presence of Z-VAD) (Figure 4.12A, $p>0.05$). This indicates that purvalanol A induced a caspase-independent change in Mcl-1 levels in LAMA-84 cell lines.

In KCL-22, purvalanol A induced a very large decrease in Mcl-1 expression by 24 h of incubation in the absence of Z-VAD. In the presence of Z-VAD, this decrease in Mcl-1 was not protected (Figure 4.12B). This confirms the caspase-independency of this decrease in protein level.

To conclude, the Mcl-1 decrease induced by purvalanol A is caspase independent in both cell lines. These results confirm the role of apoptosis induction by purvalanol A from the Viacount and cell cycle kinetic assay (Section 4.3.6.1 and 4.3.6.2).

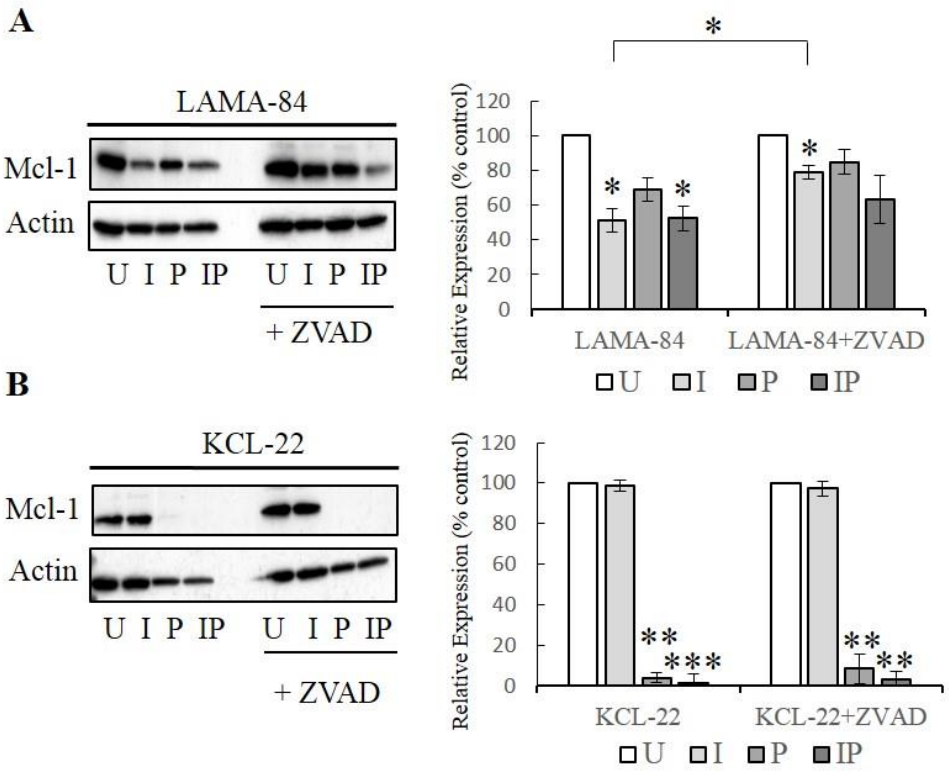

Figure 4.12 Effects of imatinib and purvalanol A on expression of Mcl-1 in LAMA-84 cells (A) and KCL-22 cells (B). Cells were incubated for 24 h in the absence (U) and presence (I) of imatinib (10 μM) or (P) purvalanol A (30 μM), ± Z-VAD (20 μM), and samples were prepared for Western blotting for Mcl-1 (40 kDa) and actin (42 kDa), quantified by densitometry. Data expressed as a % of untreated samples (±SEM, n=3), * = $p \leq 0.05$, **=$p \leq 0.01$, ***=$p \leq 0.001$ (paired two-tailed student's t-test). Western blot shown is representative from n=3 experiments.

4.3.7 Effects of purvalanol A and imatinib on intracellular signalling pathways in LAMA-84 and KCL-22 cell lines

The effects of these two inhibitors on the activation status of several intracellular signalling systems were subsequently investigated. Both LAMA-84 and KCL-22 cell lines were incubated with 30 µM purvalanol A or 10 µM imatinib for 15, 30 min and 1 h before the cell lysates were prepared and western analysis was performed to determine the effects of these drugs on CML signalling pathways. Erk, STAT3, Akt, and p38 signalling pathways were investigated in this study.

4.3.7.1 Erk signalling pathway

Imatinib completely abrogated Erk activation in LAMA-84 cells and completely decreased levels of pErk in KCL-22 cells. In contrast, purvalanol A treatment resulted in a partial decrease in pErk levels in LAMA-84 cells, but complete ablation of activation of this protein in KCL-22 cells (Figure 4.13). Purvalanol A treatment resulted in a decrease of pErk level in KCL-22 cell line by 97 ± 1.7% in 15 min ($p \leq 0.01$, n=3).

4.3.7.2 STAT3 signalling pathway

Activated STAT3 could not be detected in LAMA-84 cells in spite of the fact that this protein was expressed, as detected by pan-STAT3 antibodies. In KCL-22 cells, purvalanol A treatment decreased pSTAT3 level significantly ($p \leq 0.05$, n=3) to 22 ± 5.4 % by 15 min and completely decreased levels of pSTAT3 by 1 h of incubation (Figure 4.14).

4.3.7.3 Akt signalling pathway

Purvalanol A and imatinib treatment both decreased pAkt levels significantly in the KCL-22 cell line. The level of phosphorylated Akt was decreased by almost 100 % after every treatment for 15 min. However, pAkt was consistently undetected in LAMA-84 cell line (Figure 4.15).

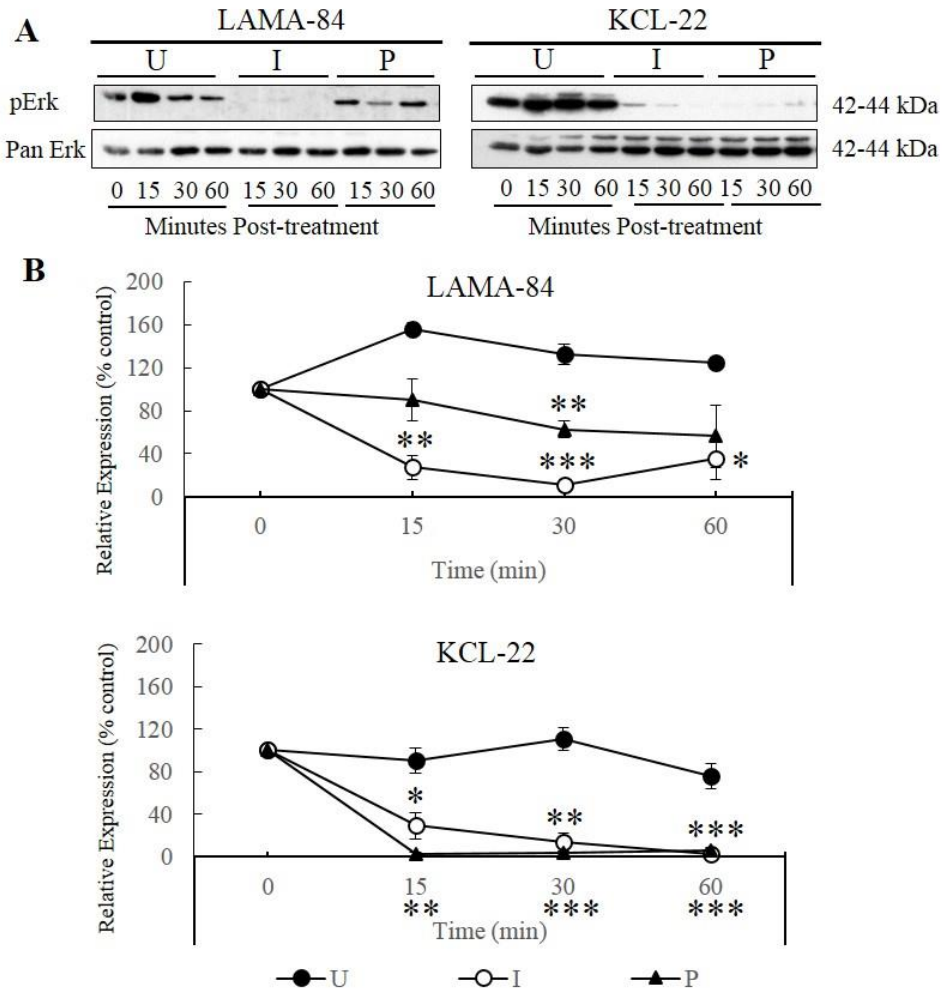

Figure 4.13 Effects of imatinib and purvalanol A on Erk activation in LAMA-84 and KCL-22 cells. Both cell lines were incubated in the absence (U ●) or presence of 10 µM imatinib (I ○) and 30 µM purvalanol A (P ▲) for 15, 30 min and 1 h before preparation for Western blotting for pErk and Pan Erk (42-44 kDa), quantified by densitometry. **(A)** shows typical western blots, while **(B)** shows densitometry analysis, expressed as a % of untreated control, untreated samples at time zero (±SEM, n=3), * = $p \leq 0.05$, **=$p \leq 0.01$, ***=$p \leq 0.001$ (One-way ANOVA followed by *Bonferroni post hoc* comparisons tests). Western blot shown is representative from n=3 experiments.

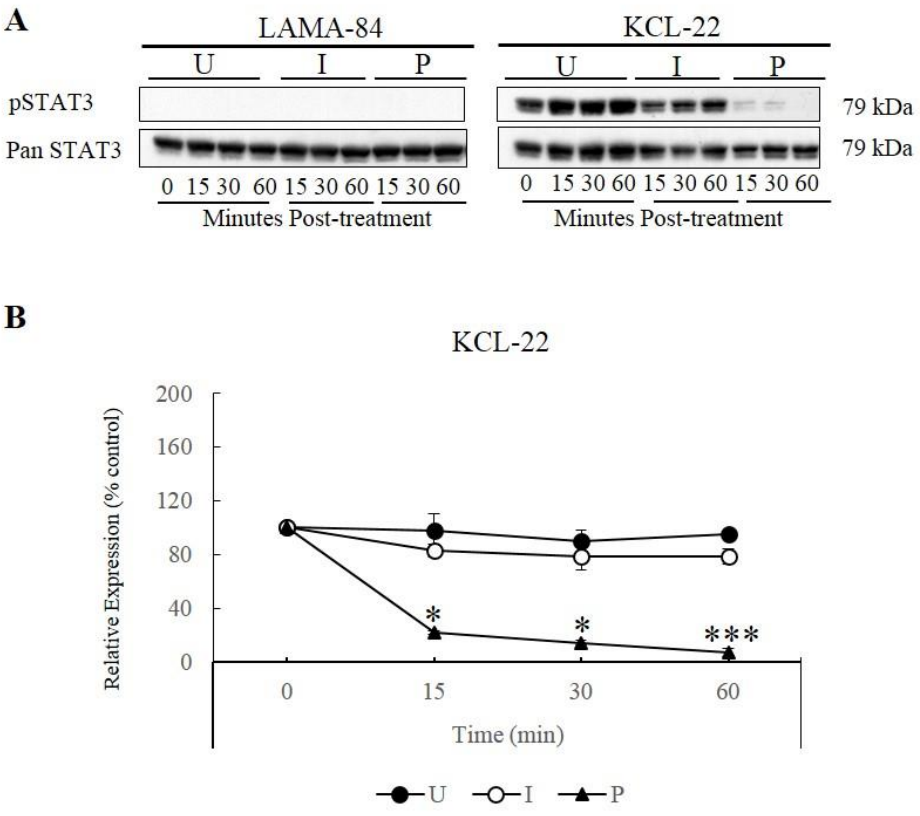

Figure 4.14 Effects of imatinib and purvalanol A on STAT3 activation in LAMA-84 and KCL-22 cells. Both cell lines were incubated in the absence (U ●) or presence of 10 µM imatinib (I ○) and 30 µM purvalanol A (P ▲) for 15, 30 min and 1 h before preparation for Western blotting for pSTAT3 and Pan STAT3 (79 kDa), quantified by densitometry. **(A)** shows typical western blots, while **(B)** shows densitometry analysis, expressed as a % of untreated control samples at time zero (±SEM, n=3), *=p≤0.05, ***=p≤0.001 (One-way ANOVA followed by *Bonferroni post hoc* comparisons tests). Western blot shown is representative from n=3 experiments.

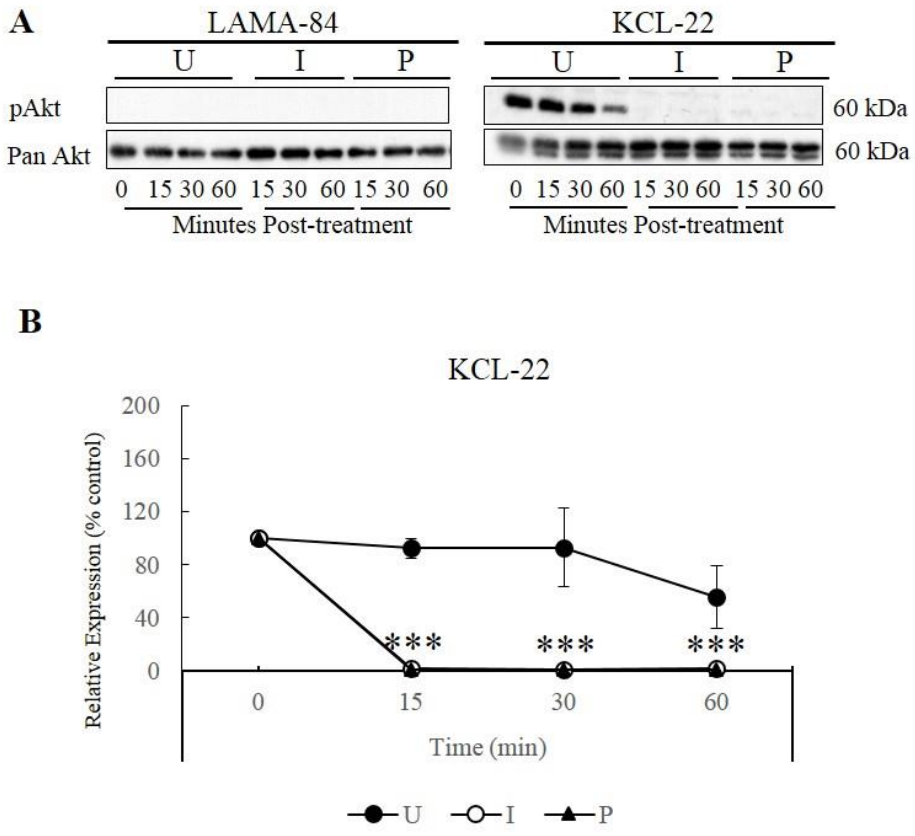

Figure 4.15 Effects of imatinib and purvalanol A on Akt activation in LAMA-84 and KCL-22 cells. Both cell lines were incubated in the absence (U ●) or presence of 10 μM imatinib (I ○) and 30 μM purvalanol A (P ▲) for 15, 30 min and 1 h before preparation for Western blotting for pAkt and Pan Akt (60 kDa), quantified by densitometry. **(A)** shows typical western blots, while **(B)** shows densitometry analysis, expressed as a % of untreated control samples at time zero (±SEM, n=3), *=p≤0.05, ***=p≤0.001 (One-way ANOVA followed by *Bonferroni post hoc* comparisons tests). Western blot shown is representative from n=3 experiments.

4.3.7.4 p38 signalling pathway

Unlike Erk and STAT3 signalling pathways, purvalanol A treatment resulted in an increase of phosphorylated p38 in both LAMA-84 and KCL-22 cell lines. Purvalanol A significantly increased phosphorylated p38 by 28 ± 6 % after 15 min incubation in KCL-22 cells. LAMA-84 cells also increased phosphorylated p38 significantly by 22 ± 17 % after 30 min (Figure 4.16).

In conclusion, imatinib completely abrogated Erk activation, but had no effect on the activation levels of p38-MAPK in LAMA-84 cells. Activated STAT3 or Akt could not be detected in LAMA-84 cells in spite of the fact that these two proteins appeared to be expressed, as detected using pan-antibodies.

In KCL-22 cells, imatinib completely decreased levels of pErk and pAkt, but had no significant effect on pSTAT3 or phospho-p38-MAPK levels. Purvalanol A treatment resulted in a partial decrease in pErk levels in LAMA-84 cells, but complete ablation of activation of this protein in KCL-22 cells, and also a complete loss of activation of pSTAT3. In complete contrast, purvalanol A treatment resulted in enhanced activation of p38-MAPK in both cell lines.

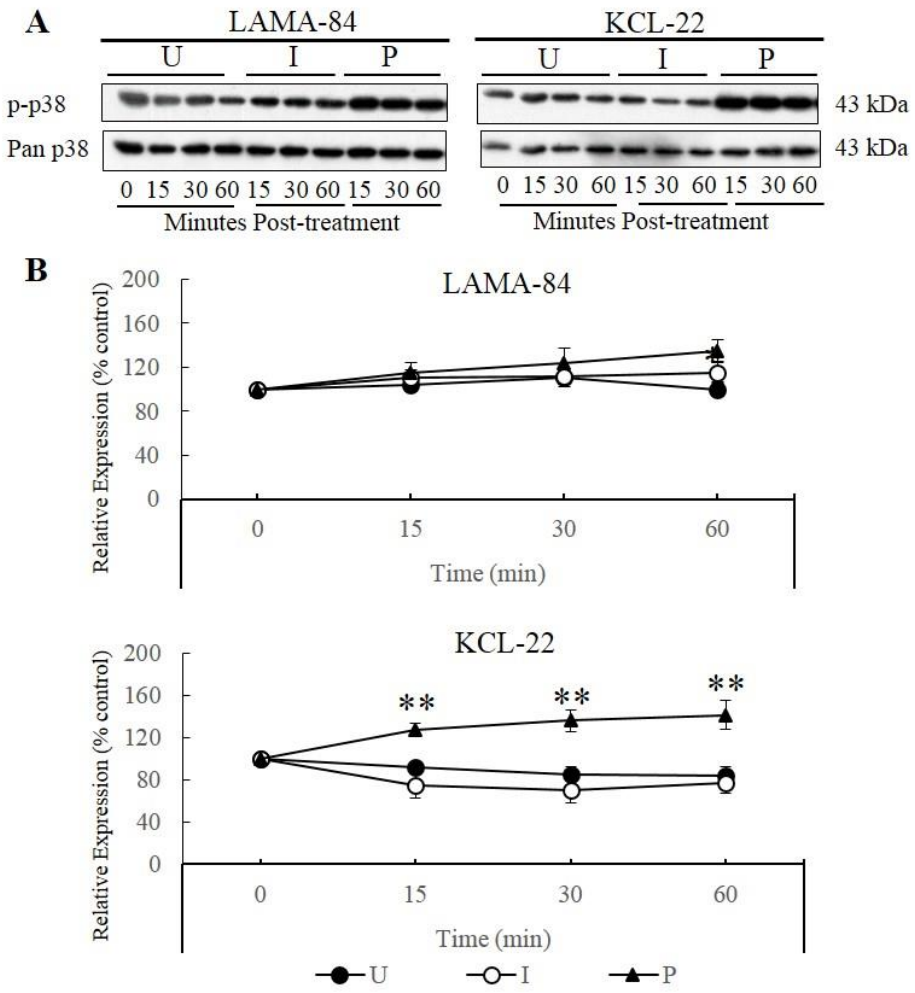

Figure 4.16 Effects of imatinib and purvalanol A on p38 activation in LAMA-84 and KCL-22 cells. Both cell lines were incubated in the absence (U ●) or presence of 10 μM imatinib (I ○) and 30 μM purvalanol A (P ▲) for 15, 30 min and 1 h before preparation for Western blotting for Phospho-p38 and Pan p38 (43 kDa), quantified by densitometry. **(A)** shows typical western blots, while **(B)** shows densitometry analysis, expressed as a % of untreated control, untreated samples at time zero (±SEM, n=3), * = p≤0.05, **=p≤0.01, ***=p≤0.001 (One-way ANOVA followed by *Bonferroni post hoc* comparisons tests). Western blot shown is representative from n=3 experiments.

4.3.8 Effects of purvalanol A and BIRB796 on the viability of LAMA-84 and KCL-22 cell lines

The above experiments show that purvalanol A activated p38-MAPK and so it is possible that activated p38 may play a role in regulating Mcl-1 expression. Therefore, the effects of BIRB796 (Kuma et al., 2005) on purvalanol induced apoptosis and Mcl-1 decrease were tested.

LAMA-84 cells and KCL-22 cells were incubated for 6 h in the absence (untreated control) and presence of 30 µM purvalanol A, 10 µM of the p38 MAPK inhibitor, BIRB796, and both inhibitors together. Cell viability was then assessed using the ViaCount assay.

Purvalanol A significantly induced apoptosis in both cell lines, ($p \leq 0.01$, $n=3$). After 6 h of incubation with purvalanol A, LAMA-84 cell viability was decreased significantly (from 94.4 ± 0.4 % cell viability of control samples to 80.6 ± 0.4 % cell viability of purvalanol A treated samples). Similarly, purvalanol A significantly decreased cell viability in KCL-22 cells line ($p \leq 0.01$, $n=3$). KCL-22 cell viability was decreased from 92.5 ± 0.2 % of control samples to 77.7 ± 1.3 % in samples treated with purvalanol A (Figure 4.17).

In contrast, BIRB796 alone did not decrease cell viability in both cell lines. However, the level of apoptosis caused by the combination of purvalanol A and BIRB796 was significantly lower than that observed by purvalanol A alone in both cell lines (to 87.2 ± 1.4 %, ($p \leq 0.05$, $n=3$) and to 88.6 ± 0.5 %, ($p \leq 0.01$, $n=3$) in LAMA-84 and KCL-22 cell line, respectively).

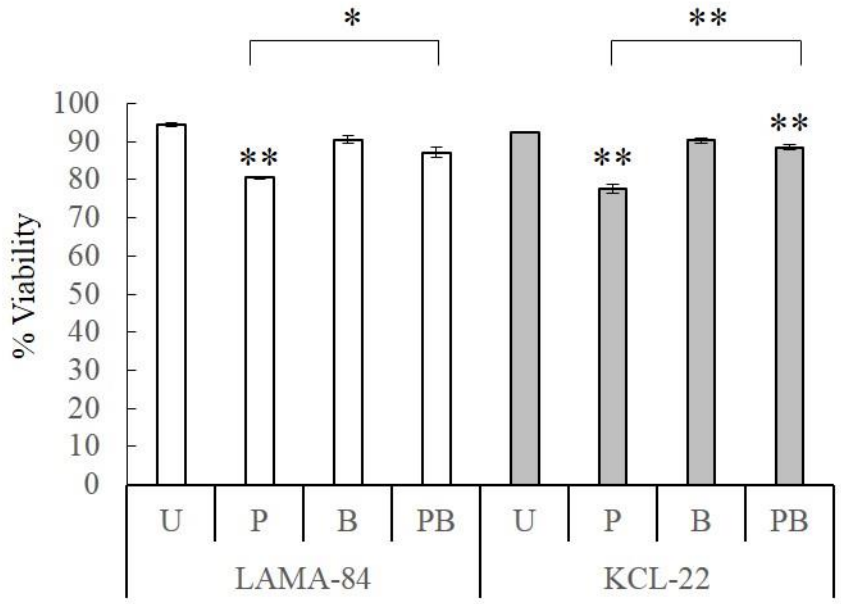

Figure 4.17 Effects of purvalanol A and BIRB796 on cell viability of CML cell lines. LAMA-84 and KCL-22 cell lines were incubated in the absence (U) and presence of 30 µM purvalanol A (P), 10 µM BIRB796 (B), and both (PB) Viability was determined using the Viacount assay. Data are shown as mean (±SEM, n=3) *= $p \leq 0.05$, **=$p \leq 0.01$ (paired two-tailed student's t-test).

4.3.9 Effects of purvalanol A and BIRB796 on expression of Mcl-1 in LAMA-84 and KCL-22 cell lines

Both cell lines were treated for 0-4 h in the absence (untreated control) and presence of 30 µM purvalanol A, 10 µM BIRB796, and both. Then, protein lysates were collected for western blotting.

In both cell lines, the co-incubation of purvalanol A with BIRB796 resulted in a significant increase of Mcl-1 protein expression compared to expression after incubation with purvalanol A alone. Densitometric analysis indicated that Mcl-1 expression was increased significantly from 66 ± 9.8 % in purvalanol A treated samples to 92.5 ± 8.3 % in purvalanol A plus BIRB796 treated samples ($p \leq 0.05$, n=3) in LAMA-84 cell line. Similarly, Mcl-1 expression in KCL-22 cell line was also increased significantly from 14.3 ± 2.6 % in purvalanol A treated samples to 32.5 ± 2.4 % in purvalanol A plus BIRB796 treated samples ($p \leq 0.05$, n=3), (Figure 4.18).

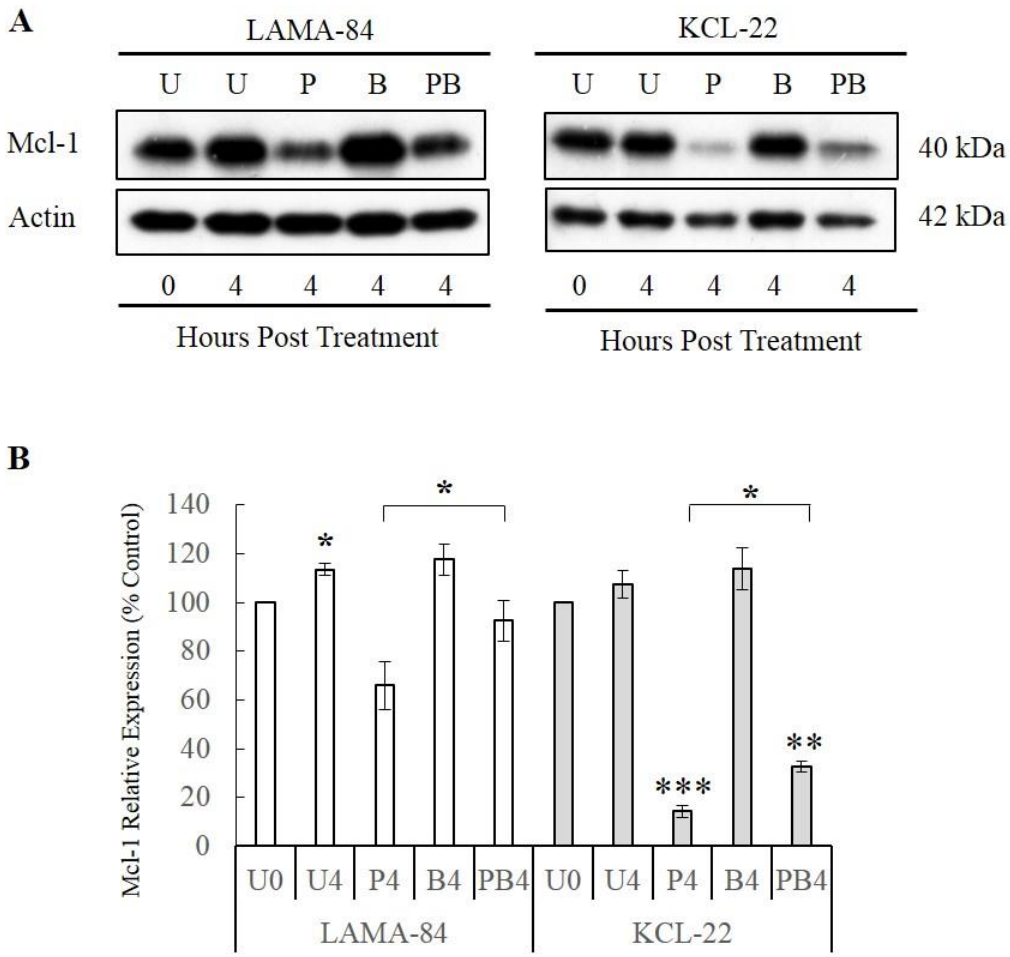

Figure 4.18 Effects of purvalanol A and BIRB796 on expression of Mcl-1 in LAMA-84 cells and KCL-22 cells. (A) Cells were incubated for 0-4 h in the absence (U) and presence 30 μM purvalanol A and (P) and 10 μM BIRB796 (B), and both inhibitors together (PB). Samples were prepared for Western blotting for Mcl-1 (40 kDa) and actin (42 kDa). Representative blots are shown in (A). **(B)** Densitometric analysis of the effects of purvalanol A and BIRB796 on Mcl-1 expression. Data are shown as mean (±SEM, n=3) *=p≤0.05, **=p≤0.01, ***=p≤0.001 (paired two-tailed student's t-test). Western blot shown is representative from n=3 experiments.

4.4 Discussion and Conclusions

In this Chapter, the effects of purvalanol A on CML viability were investigated in order to determine if this inhibitor could induce apoptosis of TKI-sensitive and –insensitive cell lines. Purvalanol A, originally developed as a CDK2 inhibitor, has been shown to inhibit CDK2 kinase activity in various cancer cell lines (Villerbu et al., 2002). However, there are no reports to date of the clinical or laboratory effects of purvalanol A on CML cells. Therefore, this study provides the first preliminary results into the effects of this inhibitor on imatinib-sensitive and –insensitive CML cell lines.

The effects of purvalanol A on LAMA-84 and KCL-22 cell lines were first investigated using cell viability measurements. This part of the study showed that purvalanol A decreased cell viability in both cell lines. Apart from the effects of purvalanol A used as a single agent in both cell lines, its effects in combination with imatinib were also investigated. However, no synergistic effects were found in these preliminary experiments, unlike in a number of other studies using inhibitors that block BCR-ABL downstream signalling pathways in combination with imatinib, some of which have found synergistic effects (Tipping et al., 2002, Radujkovic et al., 2005, Tseng et al., 2005, Kancha et al., 2008). For example, the combination of imatinib and a PDK-1 inhibitor has been shown to induce synergistic effects in imatinib-resistant cell lines (Tseng et al., 2005).

In addition to its effects on cell viability, Purvalanol A induced mitochondrial depolarisation rapidly in both LAMA-84 and KCL-22 cell lines (within 2 h). This result supports previous findings that have been reported that purvalanol A induced mitochondrial dysfunction before induction of apoptosis (Iizuka et al., 2008). The effects of purvalanol A on the expression of various pro- and anti-apoptotic proteins of the Bcl-2 family were also investigated in order to determine if changes in the expression levels of these proteins may explain the increased apoptosis observed after purvalanol A treatment. While purvalanol A induced

apoptosis in both cell lines, it induced a very large decrease in Mcl-1 levels, but only in the KCL-22 cell line. This effect was specific for Mcl-1 and was not observed for other anti-apoptotic proteins measured. This result supports the concept that Mcl-1 plays a crucial role in apoptosis of CML cells, as other reports have shown that many leukaemia cells are more sensitive to anti-Mcl-1 drugs than Bcl-2 targeted drugs (Brunelle et al., 2009). However, this result is quite different from a number of other studies using purvalanol A treatment on other cell lines to inhibit CDK2 activity. For example, studies on the human gastric adenocarcinoma cell lines and breast cancer cell lines showed that purvalanol A treatments significantly decreased the levels of Bcl-2 and Bcl-X_L in these cells (Obakan et al., 2014, Iizuka et al., 2007, Iizuka et al., 2008).

The effects of purvalanol A on the expression of the anti-apoptotic protein Mcl-1 were investigated in the presence of pan-caspase inhibitor, Z-VAD. It was found that the inhibition of caspase activity did not protect the decreases in Mcl-1 levels in both cell lines. This implies that in both cell lines, the decrease in Mcl-1 levels observed is not driven by caspase activity, an important observation because Mcl-1 degradation is reported to be caspase-sensitive under some circumstances (Weng et al., 2005, Herrant et al., 2004, Akgul, 2009). Therefore, the decreases in Mcl-1 are more liken to drive apoptosis, rather than *vice versa*. However, a previous study has shown that purvalanol A induces caspase-dependent apoptotic cell death by involving Bcl-2 family members in an aggressive Estrogen Receptor positive (ER+) breast cancer cell line, more so than in a breast cancer cell line that is Estrogen Receptor negative (ER-) (OBakan et al., 2014). This might suggest that purvalanol A has a specific effect on CML cell lines but the mechanisms of action are still unclear and the reasons for differences in responses of different cancer cells to this drug also need further investigations. It is also curious to note that while purvalanol A induced apoptosis in both cell lines, it was much more potent in inducing apoptosis in the imatinib-insensitive cell line (KCL-22) and did not result in a marked decrease in Mcl-1 in LAMA-84 cells.

Mcl-1 is subject to numerous post-translational modifications that can alter its function, localization and turnover rate (Thomas et al., 2010). Many of these post-translational modifications result from reversible phosphorylation on a number of Serine or Threonine residues, particularly in the large and unique N-terminal domain of the protein (Thomas et al., 2012). While some of the kinases responsible for these phosphorylation events have been identified (Inoshita et al., 2002, Domina et al., 2004), the precise mechanisms regulating Mcl-1 turnover are not fully defined. It is possible, therefore, that these differential effects of purvalanol A on Mcl-1 turnover in LAMA-84 and KCL-22 cells are explained, at least in part, by differences in their intracellular signalling processes that, apart from regulating cell activation could also target Mcl-1 to alter its function.

Therefore, the effects of purvalanol A on several important signalling pathways were investigated. Purvalanol A resulted in a down-regulation of phosphorylated Erk, STAT3, and Akt in KCL-22 cell line. This supports another study that used purvalanol A on different cell lines (Iizuka et al., 2008, Knockaert et al., 2002b). Moreover, this result is also related to other inhibitors on CML cell lines. For example, the proteasome inhibitor, carfilzomib, also caused a down-regulation of pERK in various types of CML models (Crawford et al., 2014). These results also support the findings that BCR-ABL activates many downstream signalling pathways, including ERK, STATs, and Akt pathways that contribute to the proliferation and survival of CML cells (Voss et al., 2000, Danial and Rothman, 2000, Kirchner et al., 2003).

However, the data presented in this Chapter revealed a number of interesting and unexpected findings. First, in spite of the fact that LAMA-84 cells appear to express STAT3 and Akt (as evidenced by the detection of an appropriate signal in western blots using pan-specific antibodies), no activated forms of these proteins could be detected under any experimental conditions tested in this study. Second, while imatinib had little effect on the growth kinetics of KCL-22 cells (apart from a partial G1 arrest), it ablated activation of both pErk and pAkt. Third, purvalanol A, but not imatinib, down-regulated STAT3 activation in KCl-22 cells. Fourth,

purvalanol A, but not imatinib resulted in increased activation of p38. Previous work has implicated p38 in neutrophil apoptosis (Aoshiba et al., 1999), and the work presented here supports the notion that activated p38 generates a death signal, perhaps directly or indirectly via increased turnover of Mcl-1. To address this question, p38 MAPK inhibitor, BIRB796, which inhibits all p38 isoforms (Kuma et al., 2005), was used with purvalanol A to determine its effect on CML cell lines. BIRB796 was found to decrease apoptosis and Mcl-1 loss caused by purvalanol A in both cell lines. This suggests that p38 MAPK plays a role in apoptosis induction by purvalanol A.

The experiments described here show that purvalanol A induced apoptosis in imatinib-insensitive cells, but the mechanism of action of purvalanol A still needs to be defined. Therefore, effects of purvalanol A on Mcl-1 will be investigated further in the next Chapter.

CHAPTER 5: EFFECTS OF PURVALANOL A ON THE EXPRESSION OF THE ANTI-APOPTOTIC PROTEIN, MCL-1, IN IMATINIB-SENSITIVE AND –INSENSITIVE CML CELL LINES

5.1 Introduction

Mcl-1 was first identified as an early induction gene expressed during myeloid leukemia cell differentiation (Kozopas et al., 1993). It was found to share amino acid sequence similarities with previously identified Bcl-2 (Reynolds et al., 1994), and to possess similar functions. It possesses three putative BH domains, which mediate anti-apoptotic functions within members of this protein family. These functions are able to delay apoptosis induced by various stimuli such as, UV-irradiation, etoposide treatment (Zhou et al., 1997) and hypoxia (Liu et al., 2006). Mcl-1 also possesses a transmembrane domain which is able to insert into many cellular membranes under resting conditions, including the mitochondria (Yang et al., 1995) and nuclear envelop (Leuenroth et al., 2000). Mcl-1 can bind to the pro-apoptotic proteins, Bak and Bax, to inhibit them from forming pores in the mitochondrial membrane. This binding inhibits cytochrome c release from mitochondria and thus can prevent apoptosis (Thomas et al., 2010).

Mcl-1 protein and mRNA have a very short half-lives (Yang et al., 1995, Schubert and Duronio, 2001) and regulation of Mcl-1 protein expression is mediated by multiple levels as described below. Mcl-1 is approximately twice as large as other Bcl-2 family members due to its extensive N-terminal domain containing PEST sequences and Arg:Arg motifs (Kozopas et al., 1993), which are responsible for the high rate of protein turnover (Rechsteiner and Rogers, 1996). Degradation of Mcl-1 can be via caspase- and proteasome-mediated mechanisms (Thomas et al., 2010).

The proteasome plays a crucial role in cellular protein degradation. More than 80% of intracellular proteins are degraded by the 26S

proteasome as it plays a role as a dynamic proteolytic complex of the ubiquitin-proteasome system. Many vital cellular processes are regulated by the proteasome, such as cell cycle kinetics, DNA repair, and apoptosis (Piccinini et al., 2003). The rapid turnover of Mcl-1 is mediated by ubiquitination at lysine residues by a Mcl-1 specific ligase, leading to proteasome degradation (Derouet et al., 2004, Chou et al., 2006). Apart from proteasome degradation, Mcl-1 can also be degraded by caspases (Herrant et al., 2004) and granzyme B (Han et al., 2005) at specific sites in the PEST regions. Thus, the short half-life characteristic of Mcl-1 can partly be explained by these protein turnover mechanisms.

Although Mcl-1 can be transcriptionally regulated, protein expression can be also regulated by post-translational regulation, as Mcl-1 residues can be phosphorylated/dephosphorylated at different sites and these modifications can alter the turnover rate or function of Mcl-1 thereby affecting its role in apoptosis (Thomas et al., 2010). Various phosphorylation sites within the PEST regions have been identified, such as Ser64 (Kobayashi et al., 2007), Ser159 (Maurer et al., 2006), Ser121 (Inoshita et al., 2002), and Ser163 (Domina et al., 2004). Phosphorylation of these site affects Mcl-1 stabilisation and subsequent Mcl-1 degradation (Thomas et al., 2010). Thus, Mcl-1 protein levels are dynamically regulated in cells and this is important for cell survival regulation.

Mcl-1 appears to have a distinct role and unique characteristics among other Bcl-2 family members, mainly due to its relatively high rate of turnover and hence transient expression. Mcl-1 expression can be rapidly regulated during development and differentiation in several cell types such as B and T lymphocytes (Opferman et al., 2003), cerebellar granular neurons (Zhang and D'Ercole, 2004), and neuroendocrinal cells (Krajewski et al., 1995). Mcl-1 knock-out in mice is embryonically lethal and this suggests it has a specific role in embryo development (Rinkenberger et al., 2000).

It has been reported that Mcl-1 over-expression in mice resulted in the development of B-cell lymphoma (Zhou et al., 2001). There are several

additional reports showing that Mcl-1 over-expression is frequently found in many types of cancer. These include multiple myeloma (Le Gouill et al., 2004), prostate cancer (Cavarretta et al., 2007), hepatocellular, carcinoma (Fleischer et al., 2006), breast cancer (Henson et al., 2006), and also CML (Aichberger et al., 2005). Aichburger and colleagues (Aichberger et al., 2005) showed that BCR/ABL promoted the expression of Mcl-1 via RAS/RAF/MAPK activation. This result suggests that Mcl-1 is an important regulator of cell survival in CML. Mcl-1 has been shown to be implicated in chemoresistant cancer (Campbell et al., 2010) and so is an interesting target in cancer therapy. Obatoclax, a BH-3 mimetic, has been developed to treat cancer (van Delft et al., 2006).

Therefore, the regulation of turnover of Mcl-1 in CML is investigated further in this chapter.

The aims of this chapter were

i) To compare the effect of the combined use of purvalanol A with imatinib or purvalanol A alone as a single agent on Mcl-1 expression in CML cell lines.

ii) To determine if the half-life of Mcl-1 is affected by purvalanol A.

iii) To measure the effects of purvalanol A on Mcl-1 mRNA.

iv) To confirm that constitutive degradation of Mcl-1 is mediated by the ubiquitin-proteasome pathway.

v) To identify the phosphorylation site of Mcl-1 that affects the stabilization of Mcl-1.

5.2 Material and Methods

All methods described in this Chapter are more fully detailed in Chapter 2. For most experiments, imatinib and purvalanol A were used at 10 μM and 30 μM, respectively, unless stated otherwise. These concentrations were the lowest concentration that induced the maximal effects on the CML cells, as used in previous Chapters. As these drugs were dissolved in DMSO, all untreated control contained an equivalent volume of DMSO as used after the addition of inhibitors.

5.3 Results

Initial experiments measured the effects of purvalanol A on Mcl-1 expression in these cell lines using western blotting. The levels of Mcl-1 mRNA expression were measured by quantitative PCR.

5.3.1 Concentration dependent effects of purvalanol A on Mcl-1 expression

Western blotting was used to determine the effects of purvalanol A on the expression levels of Mcl-1 in KCL-22 cells, which were treated with and without purvalanol A over a range of different concentrations (2.5, 5, 10, 20, and 30 μM) in the presence and absence of imatinib (10 μM) for 24 h and then protein lysates were prepared for western blotting. After 24 h incubation with purvalanol A in the absence of imatinib, Mcl-1 levels decreased significantly at concentrations of 20 μM (56.8 ± 9.7 %) and 30 μM (17.5 ± 12.9 %) compared to untreated control ($p \leq 0.05$, n=3).

However, in the presence of imatinib, the Mcl-1 levels were significantly decreased at the concentration of purvalanol A at 10 μM (58 ± 8 %), 20 μM (19.8 ± 6.3 %), and 30 μM (1 ± 0.4 %) ($p \leq 0.05$, n=3).

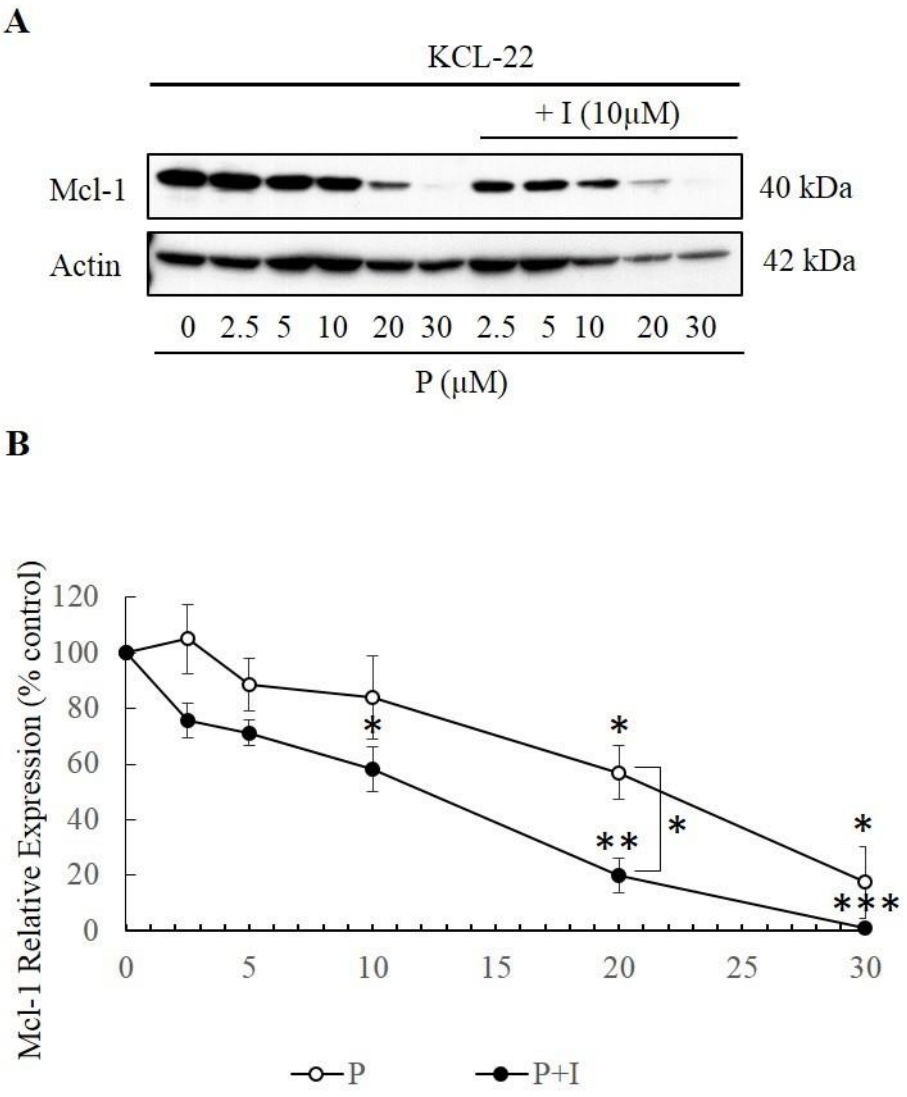

Figure 5.1 Effect of different concentrations of purvalanol A on expression of Mcl-1 in KCL-22 cells. Cells were incubated in the absence and presence of purvalanol A (P) at concentrations of 0, 2.5, 5, 10, 20, and 30 μM with and without imatinib (10 μM) for 24 h and samples were prepared for Western blotting for Mcl-1 (40 kDa) and actin (42 kDa), quantified by densitometry. **(A)** Shows a typical western blot analysis, while densitometric analysis is shown in **(B).** Data are expressed as % of untreated samples (±SEM, n=3) * = p≤0.05, ** = p≤0.01, *** = p≤0.001 (paired two-tailed student's t-test).

The combination of purvalanol A and imatinib decreased Mcl-1 expression in KCL-22 cells more than purvalanol A alone at the equivalent concentration. These results showed that there was a significant difference between the incubation of purvalanol A with the presence and absence of imatinib at the 20 μM concentration of purvalanol A ($p \leq 0.05$, $n=3$) (Figure 5.1).

In addition, the combination of purvalanol A and other TKIs (nilotinib and dasatinib) showed similar effect to imatinib. The combination treatment showed greater effect in Mcl-1 level reduction when compared to single treatment (Figure 5.2 and 5.3).

5.3.2 Time course of effects of purvalanol A on Mcl-1 expression

Subsequent experiments were then performed to measure the time course of the effects of imatinib and purvalanol A on Mcl-1 expression. LAMA-84 and KCL-22 cell lines were treated with purvalanol A for 0, 1, 2, 4, 6, 18, and 24 h before protein extracts were prepared for western blot analysis. The results showed that purvalanol A decreased Mcl-1 levels rapidly in KCL-22 cells, but not LAMA-84 cells. In KCL-22 cells, significantly decreased Mcl-1 was observed by 1 h (83 ± 7.5 %) of purvalanol A incubation ($p \leq 0.05$, $n=3$) (Figure 5.4). In addition, purvalanol A decreased Mcl-1 expression by 50 % of the time zero values within approximately 4 h of incubation (Figure 5.4).

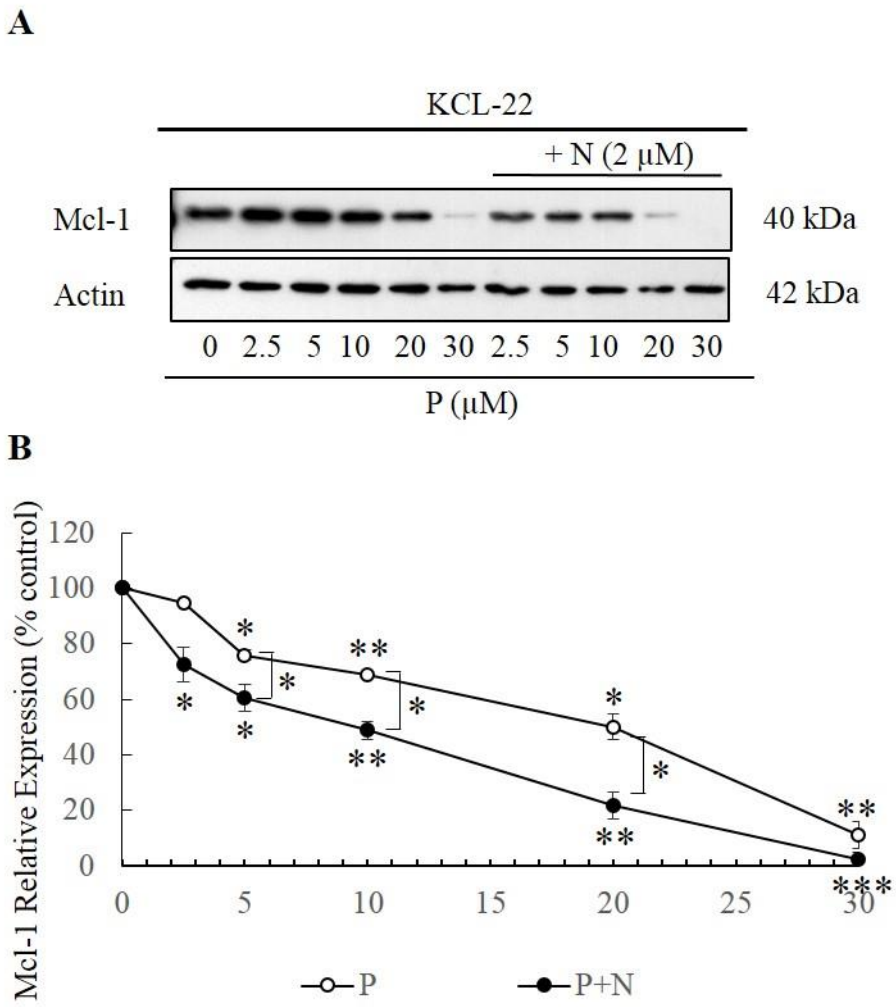

Figure 5.2 Effect of different concentrations of purvalanol A and nilotinib on expression of Mcl-1 in KCL-22 cells. Cells were incubated in the absence and presence of purvalanol A (P) at concentrations of 0, 2.5, 5, 10, 20, and 30 µM with and without nilotinib (2 µM) (N) for 24h and samples were prepared for Western blotting for Mcl-1 (40 kDa) and actin (42 kDa), quantified by densitometry. **(A)** Shows a typical western blot analysis, while densitometric analysis is shown in **(B).** Data are expressed as % of untreated samples (±SEM, n=3), * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ (paired two-tailed student's t-test).

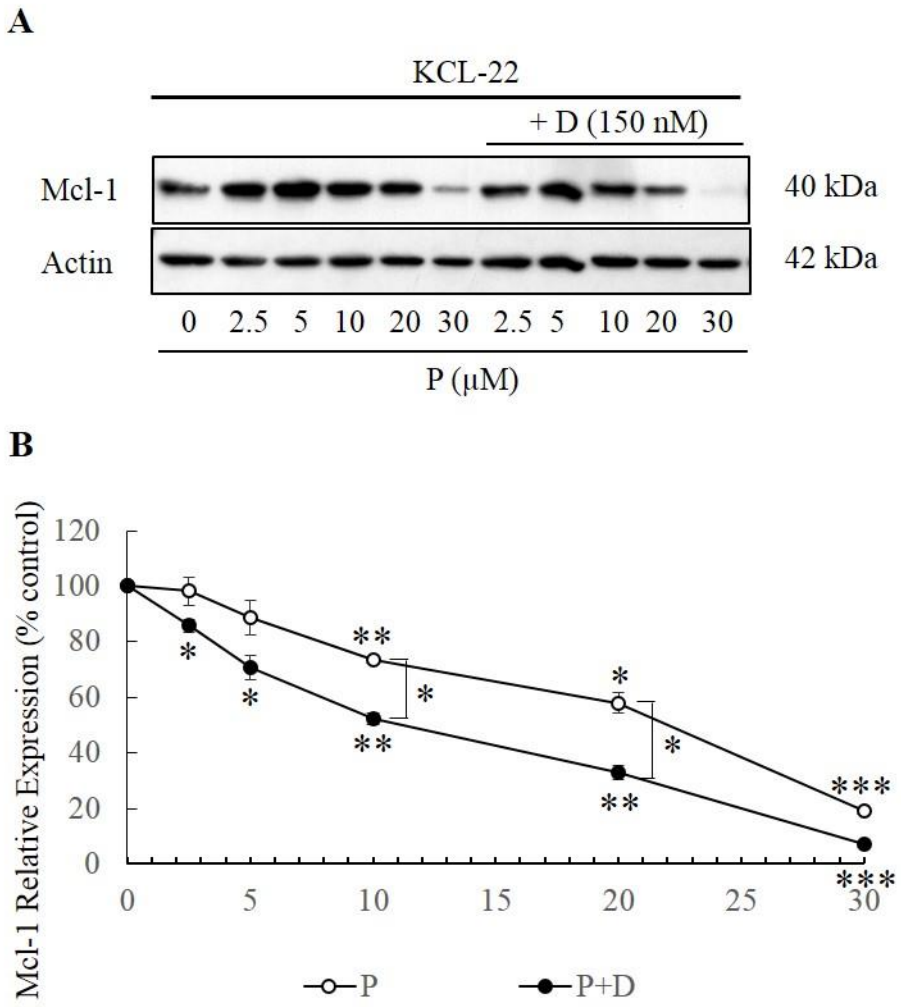

Figure 5.3 Effect of different concentrations of purvalanol A and dasatinib on expression of Mcl-1 in KCL-22 cells. Cells were incubated in the absence and presence of purvalanol A (P) at concentrations of 0, 2.5, 5, 10, 20, and 30 μM with and without Dasatinib (150 nM) (D) for 24h and samples were prepared for Western blotting for Mcl-1 (40 kDa) and actin (42 kDa), quantified by densitometry. **(A)** Shows a typical western blot analysis, while densitometric analysis is shown in **(B).** Data are expressed as % of untreated samples (±SEM, n=3), * = p≤0.05, ** = p≤0.01, *** = p≤0.001 (paired two-tailed student's t-test).

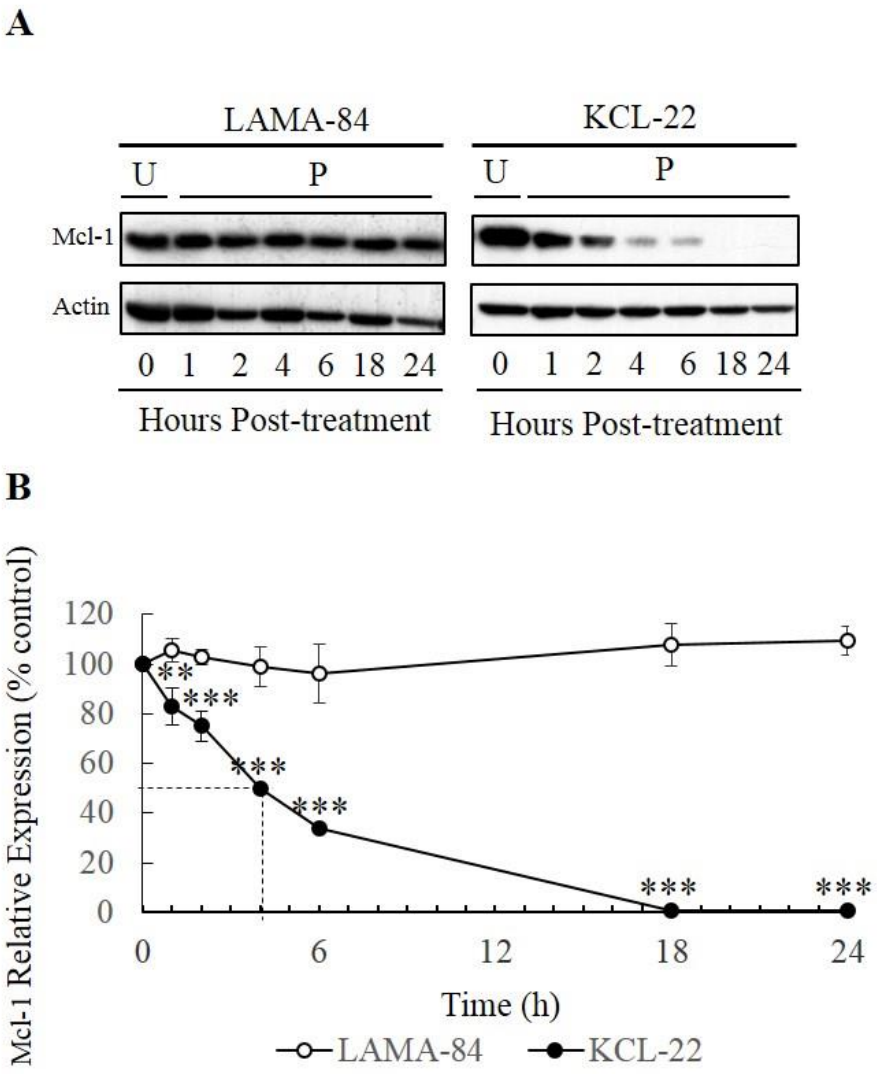

Figure 5.4 Time course of effects of purvalanol A on expression of Mcl-1 in LAMA-84 cells and KCL-22 cells. Cells were incubated in the absence and presence of purvalanol A (30 μM), and samples were collected at various incubation times and prepared for Western blotting for Mcl-1 (40 kDa) and actin (42 kDa), quantified by densitometry. **(A)** Shows a typical western blot analysis, while densitometric analysis of LAMA-84 and KCL-22 cell lines is shown in **(B).** Data expressed as a % of untreated samples (±SEM, n=3) * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ (paired two-tailed student's t-test).

5.3.3 Effect of purvalanol A on the half-life of Mcl-1

Mcl-1 has a short half-life that can be decreased even further in the presence of certain apoptosis-inducing agonists, or enhanced by agents that delay apoptosis, such as GM-CSF (Derouet et al., 2004). The following experiments were performed to determine if purvalanol A induced apoptosis via changes in the turnover rate of Mcl-1. Cells were incubated with the *de novo* protein synthesis inhibitor, cycloheximide (CHX), prior to purvalanol A treatment. After this pre-incubation period, the cycloheximide-treated cells were incubated for 1, 2, 4, and 6 h in the absence and presence of 30 μM of purvalanol A, and protein extracts were prepared. Levels of Mcl-1 protein in these cells that were blocked in protein synthesis were determined by western blotting.

These results show that when *de novo* protein synthesis was blocked, Mcl-1 was constitutively degraded and this turnover was accelerated in the presence of purvalanol A in KCL-22, but not LAMA-84 cells.

In control cells (cycloheximide but no purvalanol A), the half-life of Mcl-1 (the time taken for the level of Mcl-1 to decrease by 50 % of time zero values in cycloheximide-blocked cells) was ~3 h in both LAMA-84 cells and KCL-22 cells. Purvalanol A resulted in only a slight change in the half-life of Mcl-1 in LAMA-84 cells (Figure 5.5), but a large decrease in the half-life of Mcl-1 in KCL-22 cells to approximately 1.5 h (Figure 5.6).

Mcl-1 levels in KCL-22 were significantly lower after 4 h after CHX treatment in the presence of purvalanol A incubation (20.1 ± 5.3 %) compared to treatment with CHX alone (42.1 ± 2.7) ($p \leq 0.05$, $n=3$). These experiments indicate that the very rapid decrease in Mcl-1 levels in KCL-22 cells induced by purvalanol A treatment is explained, at least in part, by an increase in the turnover rate of Mcl-1.

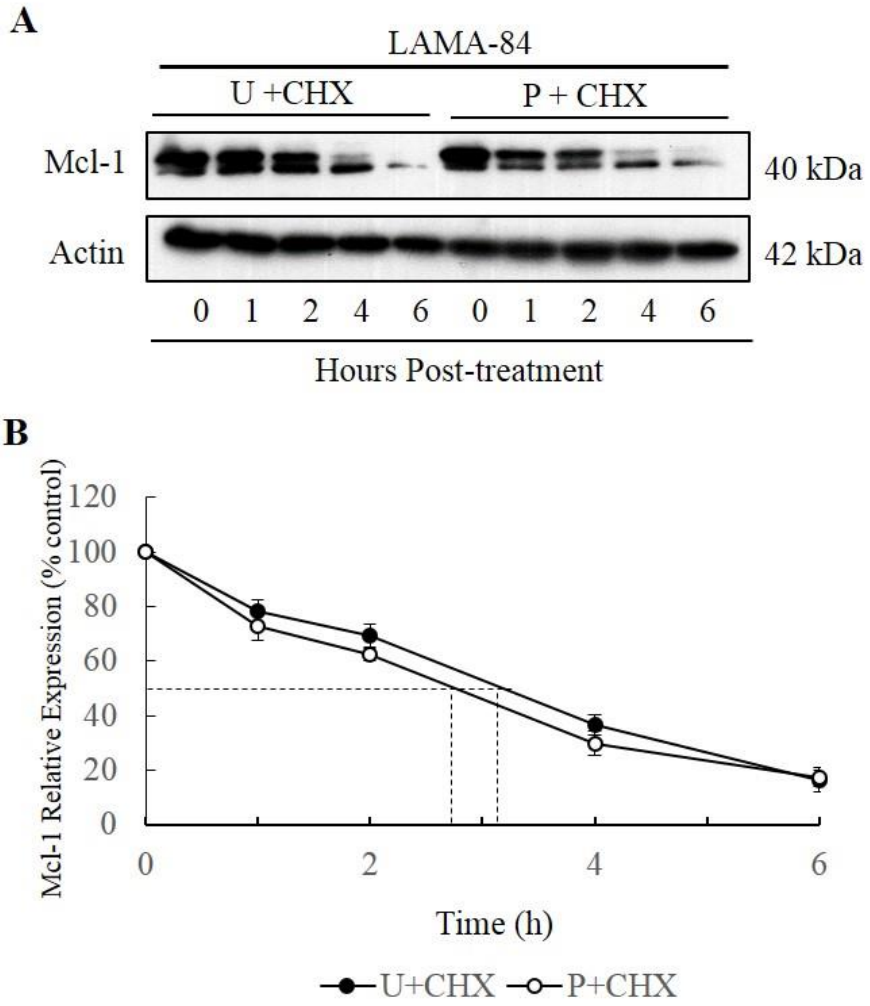

Figure 5.5 Effect of purvalanol A on the half-life of Mcl-1 in LAMA-84 cells. Cell lines were pre-incubated for 10 min with cycloheximide (CHX) prior to 0, 1, 2, 4, and 6 h incubation in the absence (U), or presence of 30 μM purvalanol A (P). Cell samples were collected at various times and prepared for Western blotting for the levels of Mcl-1 (40 kDa) and actin (42 kDa), which were quantified by densitometry. **(A)** Shows a typical western blot analysis, while densitometric analysis is shown in **(B).** The half-life of untreated samples and purvalanol A treated samples were indicated with IC_{50} (shown in dashes). Data are expressed as % of untreated samples (±SEM, n=3). Western blot shown is representative from n=3 experiments.

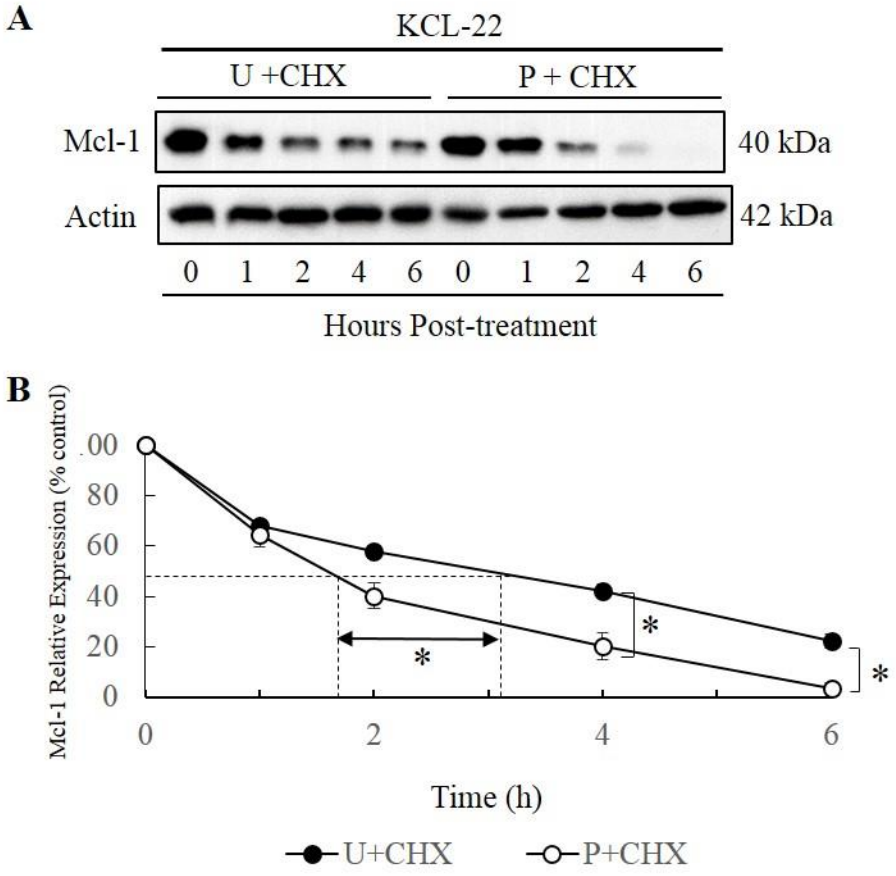

Figure 5.6 Effect of purvalanol A on the half-life of Mcl-1 in KCL-22 cells. Cell lines were pre-incubated for 10 min with cycloheximide (CHX) prior to 0, 1, 2, 4, and 6 h incubation in absence (U), or presence of 30 μM purvalanol A (P). Cell samples were collected at various times and prepared for Western blotting for the levels of Mcl-1 (40 kDa) and actin (42 kDa), were quantified by densitometry. **(A)** Shows a typical western blot analysis, while densitometric analysis is shown in **(B).** The half-life of untreated samples and purvalanol A treated samples were indicated with IC_{50} (shown in dashes). Data are expressed as % of untreated samples (±SEM, n=3), * = $p \leq 0.05$ (paired two-tailed student's t-test). Western blot shown is representative from n=3 experiments.

5.3.4 Effect of purvalanol A on Mcl-1 mRNA expression

The effects of purvalanol A on Mcl-1 mRNA expression and mRNA turnover were then determined in view of the finding in previous experiments which showed that purvalanol A caused an enhanced rate of Mcl-1 turnover in imatinib-resistant cells. LAMA-84 and KCL-22 cells were treated with and without 30 µM purvalanol in the presence and absence of 10 µM imatinib for 30 min and 2 h and then total RNA was isolated for cDNA synthesis and used to perform qPCR.

In LAMA-84 cells, imatinib and purvalanol A had no significant effect on the relative expression of Mcl-1 mRNA (Figure 5.7).

KCL-22 cells showed a different response in Mcl-1 mRNA expression after purvalanol A exposure. The relative expression of Mcl-1 mRNA was not affected by imatinib over a period of 2 h incubation. In contrast, Mcl-1 mRNA was significantly down-regulated after 30 min exposure to purvalanol A (63.9 ± 7.3 %). The combination of imatinib and purvalanol A also significantly decreased the relative expression of Mcl-1 mRNA after a period of 2 h incubation (55.5 ± 6.3 %), ($p \leq 0.05$, n=3) (Figure 5.7). The maximal decrease in Mcl-1 mRNA expression was detected by 2 h incubation (39.2 ± 19.6 %) after purvalanol A treatment of KCL-22 cells ($p \leq 0.05$, n=3).

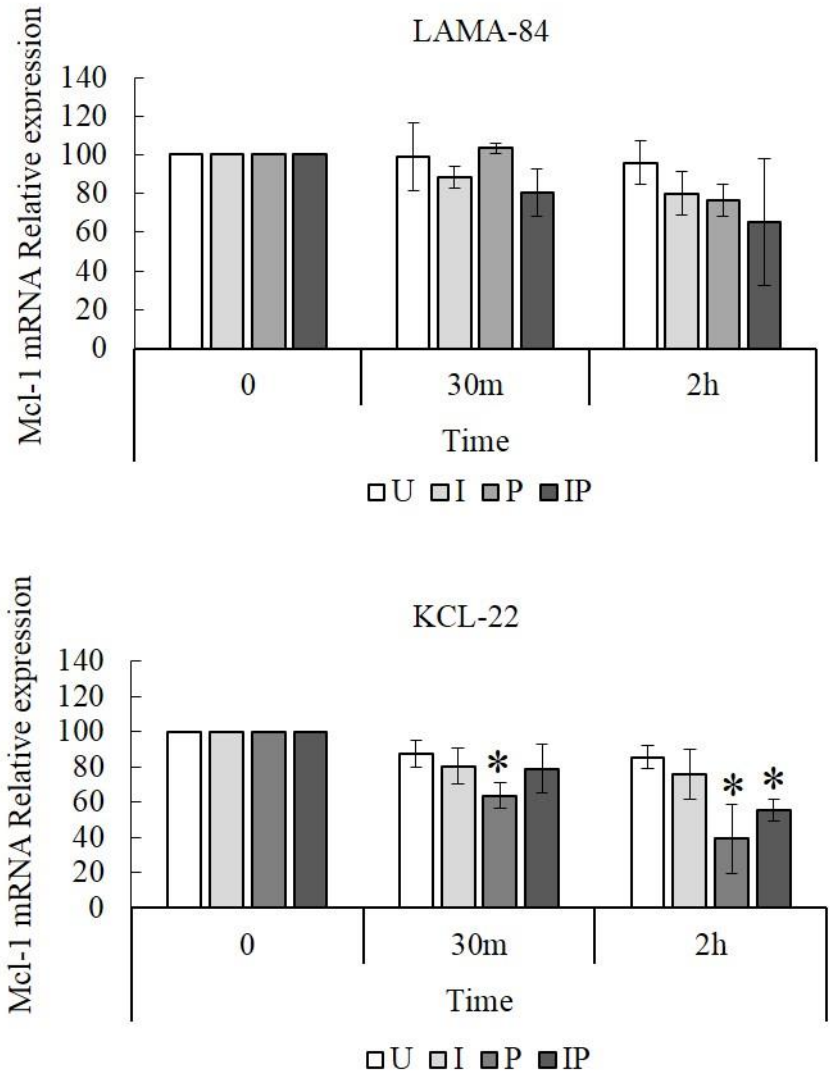

Figure 5.7 Effect of imatinib and purvalanol A on mRNA expression of Mcl-1 in LAMA-84 (A) and KCL-22 cells (B). Cell lines were incubated in absence (U), or presence of 10 μM imatinib (I), 30 μM purvalanol A (P), and a combination of the two drugs (IP) for 0, 30 min and 2 h incubation. Cell samples were collected and prepared for RNA isolation, followed by quantitative PCR for the levels of Mcl-1L and β-actin. Data are expressed as % of untreated samples (±SEM, n=3), * = $p \leq 0.05$ (paired two-tailed student's t-test).

5.3.5 Effect of purvalanol A and proteasome inhibition on Mcl-1 protein expression

The above results suggest that purvalanol A induced apoptosis in KCL-22 cell line by increasing Mcl-1 protein turnover. Thus, it was necessary to determine whether purvalanol A decreased Mcl-1 expression in KCL-22 cells by proteasome degradation and promoting ubiquitination.

To address this question, both LAMA-84 and KCL-22 cells were incubated for 2 h in the absence (U) and presence of 30 μM purvalanol A (P) with and without 10 μM cycloheximide (C) and 50 μM of the proteasome inhibitor, MG-132 (M). After this incubation, samples were collected and prepared for western blotting for Mcl-1 (40 kDa) and actin (42 kDa).

In LAMA-84 cells, the level of Mcl-1 protein were always higher in the presence of MG-132, than in the absence of this proteasome inhibitor. There was a significant difference in Mcl-1 expression between purvalanol A treated samples (P as 81.5 ± 7.2 %) and purvalanol A together with MG-132 (MP as 107.7 ± 5.3 %), ($p \leq 0.05$, n=3) (Figure 5.8). This suggests that turnover of Mcl-1 in the presence of purvalanol A occurs via the proteasome. In addition, over-exposed western blots in Figure 5.10A indicate the presence of high molecular mass bands of Mcl-1 in MG-132 cells incubated together with purvalanol A (b) than when incubated with purvalanol A alone (a). These higher molecular mass forms of Mcl-1 may be ubiquitinated forms of the protein in the presence of the proteasome inhibitor, MG-132.

Similar to LAMA-84 cells, MG-132 prevented the loss of Mcl-1 in KCL-22 cells when exposed to purvalanol A. The Mcl-1 expression in cells treated with MG-132 together with purvalanol A (MP as 115.4 ± 3.7 %) was significantly higher that purvalanol A alone (P as 47.8 ± 3.6 %), ($p<0.05$, n=3) (Figure 5.9). Also, over-exposed western blots (Figure 5.10B) shows more higher molecular mass forms of Mcl-1 band in the presence of MG-132 in purvalanol A treated samples (d) compared to purvalanol A alone (c).

To conclude, these results suggest that purvalanol A promoted ubiquitination and proteasome degradation in both LAMA-84 and KCL-22 cell lines.

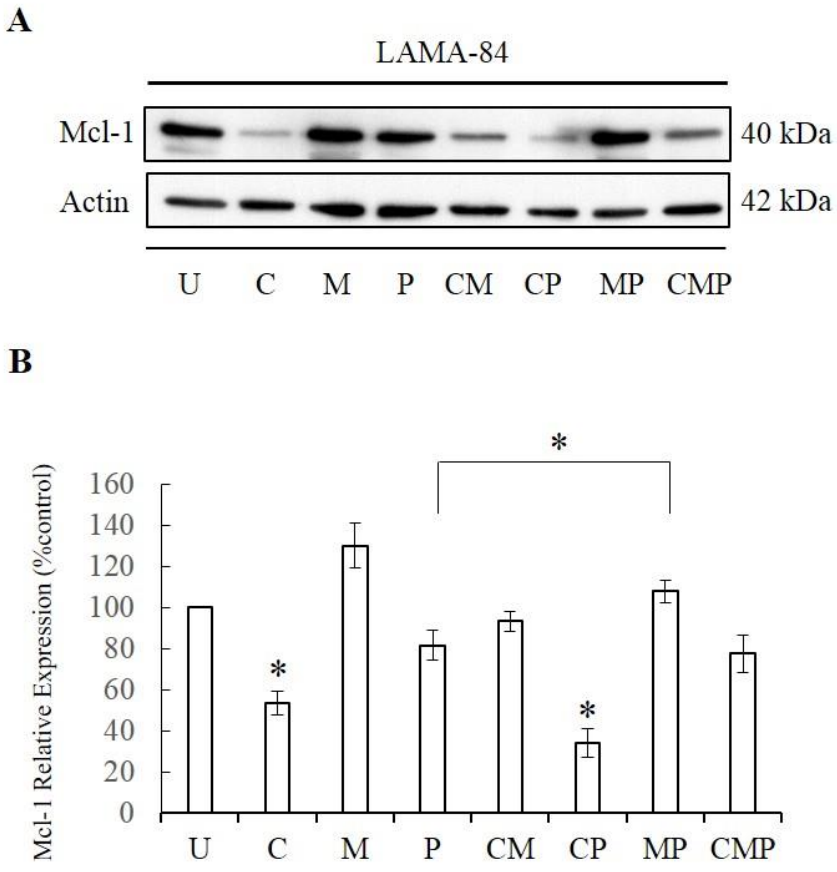

Figure 5.8 Effect of purvalanol A and proteasome inhibitor on Mcl-1 protein expression in LAMA-84. Cells were incubated in the absence (U) and presence of purvalanol A (P) (30 μM) with and without cycloheximide (C) and MG-132 (M) for 2 h, and samples were collected and prepared for Western blotting for Mcl-1 (40 kDa) and actin (42 kDa), quantified by densitometry. **(A)** Shows a typical western blot analysis, while densitometric analysis is shown in **(B).** Data expressed as a % of untreated samples (±SEM, n=3), * = $p \leq 0.05$ (paired two-tailed student's t-test). Western blot shown is representative from n=3 experiments.

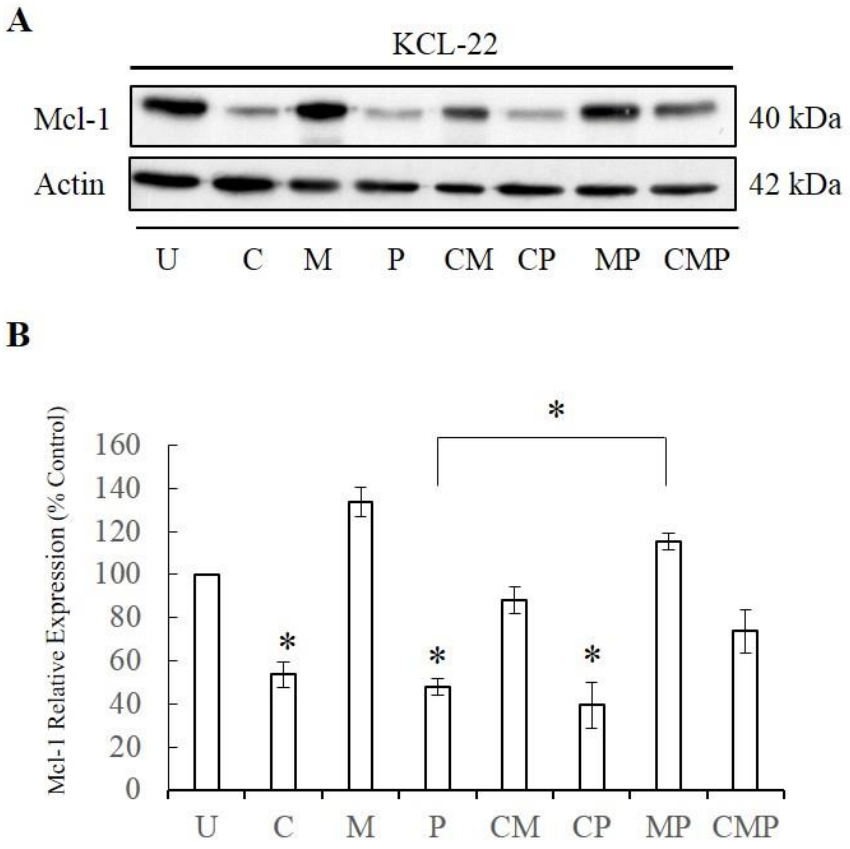

Figure 5.9 Effect of purvalanol A and proteasome inhibitor on Mcl-1 expression in KCL-22. Cells were incubated in the absence (U) and presence of purvalanol A (P) (30 μM) with and without cycloheximide (C) and MG-132 (M) for 2 h, and samples were collected and prepared for Western blotting for Mcl-1 (40 kDa) and actin (42 kDa), quantified by densitometry. **(A)** Shows a typical western blot analysis, while densitometric analysis is shown in **(B).** Data expressed as a % of untreated samples (±SEM, n=3), * = $p \leq 0.05$ (paired two-tailed student's t-test). Western blot shown is representative from n=3 experiments.

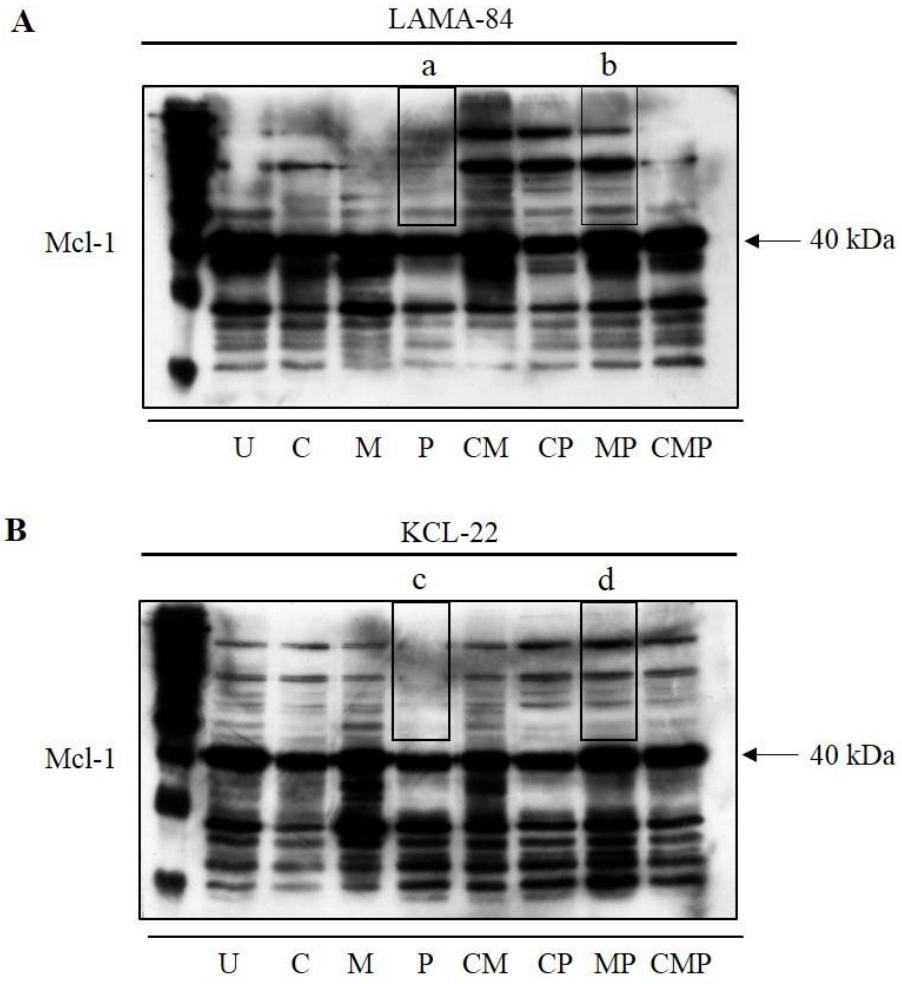

Figure 5.10 Effects of purvalanol A and proteasome inhibitor on Mcl-1 expression in LAMA-84 and KCL-22. Cells were incubated in the absence (U) and presence of purvalanol A (P) (30 μM) with and without cycloheximide (C) and MG-132 (M) for 2 h, and samples were collected and prepared for Western blotting for Mcl-1 (40 kDa) and actin (42 kDa), quantified by densitometry (films were over-exposed to membranes for at least 10 min). **(A)** Shows a typical western blot analysis of LAMA-84 cells, while a typical western blot analysis of KCL-22 cells is shown in **(B)**. (a-d) indicates higher molecular mass forms of Mcl-1. Western blot shown is representative from n=3 experiments.

5.3.6 Effect of purvalanol A on phospho-Mcl-1 expression

The above experiments showed that purvalanol A induced apoptosis by an increase of Mcl-1 turnover, possibly involving enhanced ubiquitination, and subsequent proteasome degradation. Recent work suggests that phosphorylation of Mcl-1 may promote ubiquitination (Inuzuka et al., 2011, Magiera et al., 2013). Thus, levels of phospho-Mcl-1 expression were investigated to address this question. Both LAMA-84 and KCL-22 cells were incubated for 2 h in the absence (U) and presence of 30 μM purvalanol A (P) with and without 10 μM imatinib (I). Cell lysates were collected and prepared for western blotting for phospho-Mcl-1 (Ser 159 / Thr 163) (40 kDa), Mcl-1 (40 kDa), and actin (42 kDa).

There was no significant difference observed in p-Mcl-1 expression after any treatment in LAMA-84 cell line. Figure 5.11A shows a typical western blot analysis of p-Mcl-1. p-Mcl-1 appeared as a double band at around 40 kDa and Figure 5.11C illustrates the ratio of the relative expression of the upper band and the lower band (to indicate hyper phosphorylation). In LAMA-84 cells, the relative expression of the upper band (p-Mcl-1) was significantly higher than the lower band ($p \leq 0.05$, $n=3$). This suggests that purvalanol A may induce p-Mcl-1 (Ser 159 / Thr 163) hyper phosphorylation in these cells.

In contrast, purvalanol A decreased p-Mcl-1 expression significantly in KCL-22 cell line (P = 29.4 ± 9.3 %, IP = 40.9 ± 8.7 %, compared to untreated controls (U), $p \leq 0.05$, $n=3$), while imatinib alone did not have the same effect. However, there was no significant difference in the ratio between the relative expression p-Mcl-1 as determined by the ratio of the upper and lower band of p-Mcl-1.

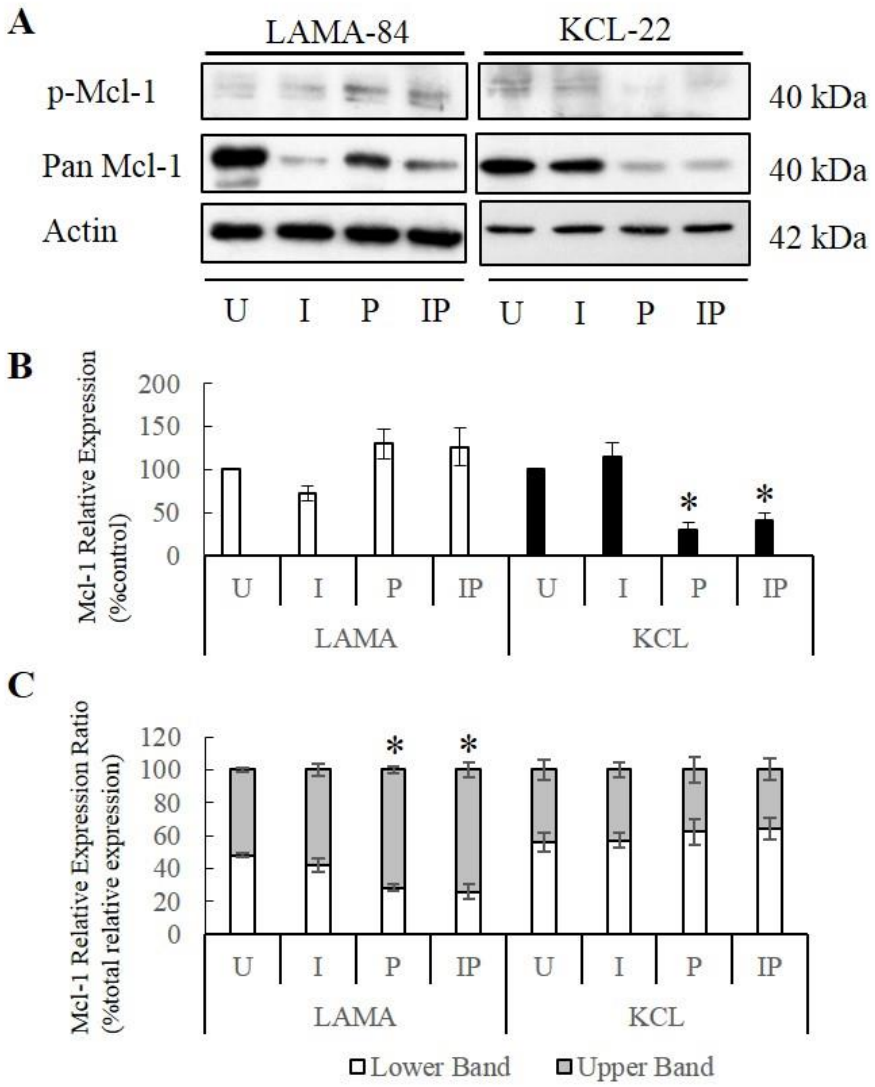

Figure 5.11 Effects of purvalanol A on phospho-Mcl-1 expression. Cells were incubated in the absence (U) and presence of purvalanol A (P) (30 μM) with and without imatinib (10 μM) (I) for 2 h, and samples were collected and prepared for Western blotting for p-Mcl-1 (40 kDa), Mcl-1 (40 kDa), and actin (42 kDa). **(A)** Shows a typical western blot analysis, while densitometric analysis of p-Mcl-1 quantified by Pan Mcl-1 is shown in **(B)**. **(C)** shows the ratio of relative expression between upper and lower band. Data expressed as a % of untreated samples (±SEM, n=3), * = $p \leq 0.05$ (paired two-tailed student's t-test). Western blot shown is representative from n=3 experiments.

5.4 Discussion and Conclusions

Mcl-1 expression is implicated in drug resistance in various types of cancers, such as leukaemias (van Delft et al., 2006). A recent report has shown that Mcl-1 transgenic mice over-expressing Mcl-1 developed lymphopoeisis that was refractory to chemotherapy (Campbell et al., 2010). Thus, there is a need to find new ways to decrease the level of Mcl-1 expression, as this may have potential to function as an alternative therapy for cancer treatment.

Mcl-1 protein and mRNA have a very short half-lives (Yang et al., 1995, Schubert and Duronio, 2001) which can be decreased even further in the presence of certain apoptosis-inducing agonists (Derouet et al., 2006). This characteristic suggests that the level of Mcl-1 expression is highly regulated by transcription and/or translation inhibition. This study confirms the hypothesis that purvalanol A induced apoptosis in imatinib-insensitive cell line by enhancing Mcl-1 turnover. This result supports the concept that Mcl-1 plays a crucial role in apoptosis of CML cells (Aichberger et al., 2005). There are several reports supporting this concept that Mcl-1 can be targeted by many agents that affect its turnover. For example, Seliciclib, a CDK inhibitor, can induce RNA polymerase II-dependent transcription, which results in Mcl-1 degradation and induction of apoptosis (MacCallum et al., 2005). Moreover, it has been reported that quercetin, a plant flavonoid, induced apoptosis in U-937 cell lines via Mcl-1 down-regulation (Cheng et al., 2010).

Mcl-1 can be regulated indirectly by alterations in its turnover rate by some agents. For example, obatoclax, the BH3-like compound, can interfere with the interaction between Mcl-1 and the pro-apoptotic protein, Bak and this leads to cell apoptosis (Nguyen et al., 2007, Trudel et al., 2007). The work presented in this Chapter suggests that purvalanol A can be included in this group of compounds as it increases Mcl-1 turnover in imatinib-insensitive CML cell lines. However, its mechanism of action should be investigated further. Apart from its effects on increasing protein turnover, the decrease of Mcl-1 expression in imatinib-insensitive cells

could also be partly explained by effects on transcriptional and/or post transcriptional inhibition. Thus, the effects of purvalanol A on Mcl-1 mRNA expression were determined by qPCR. The experiments described in this Chapter shows that purvalanol A decreased Mcl-1 mRNA levels in imatinib-resistant cells in a time-dependent manner. Thus, the effects of purvalanol A on Mcl-1 are of potential therapeutic interest, as Mcl-1 can be inhibited at transcriptional level, leading to a decrease in Mcl-1 expression in imatinib-insensitive CML cells.

This study also suggests that purvalanol A promoted ubiquitination prior to proteasome degradation in both imatinib-sensitive and –insensitive CML cell lines. This supports the concept that Mcl-1 degradation depends on proteasome-dependent mechanisms (Thomas et al., 2010). This is because the N-terminal region of Mcl-1 contains PEST domains that contain motifs that can regulate protein degradation. This PEST region contains ubiquitination sites (lysine residues) that can promote proteasome degradation (Thomas et al., 2010, Akgul, 2009). This suggests that purvalanol A has different modes of actions on Mcl-1 expression in addition to Mcl-1 mRNA down-regulation. To support this concept, it has been reported that quercetin down-regulated Mcl-1 by disturbing mRNA stability and enhancing proteasome degradation in CLL cells (Spagnuolo et al., 2011). Also, another report showed that the Mcl-1 down-regulation was mediated by a proteasomal-dependent pathway in the presence of certain apoptosis-inducing agent in human neutrophils (Lucas et al., 2013).

In this Chapter, some preliminary experiments on Mcl-1 phosphorylation are provided. The experiments described here showed that purvalanol A decreased p-Mcl-1 expression (Ser159 / Thr163) in imatinib-insensitive cells. This supports the concept that Mcl-1 can be post-translationally regulated by phosphorylation at specific residues on Mcl-l. In this study, the potential phosphorylation sites that were studied using the antibody were at Ser159 and Thr163. These phosphorylated sites have been experimentally confirmed (Maurer et al., 2006, Inoshita et al., 2002, Domina et al., 2004). It has been reported that phosphorylation at Thr163 increases Mcl-1 stability by delaying its turnover (Domina et al., 2004).

However, another report shows that phosphorylation at this site induced Mcl-1 inactivation after H_2O_2 treatment (Inoshita et al., 2002). Thus, the role of Thr163 phosphorylation is still unclear and need to be investigated further.

Taken together, these results provide new insights into how Mcl-1 turnover is regulated by purvalanol A. These include Mcl-1 mRNA down-regulation, ubiquitination and proteasome degradation, and protein phosphorylation. However, more research, including more *in vitro* experiments, need to be performed to determine the effects of purvalanol A on the Mcl-1 in CML cells and primary immune cells, such as PBMCs and neutrophils.

CHAPTER 6: EFFECTS OF PURVALANOL A ON PRIMARY LEUKOCYTES

6.1 Introduction

White blood cells (leukocytes) protect the human body from pathogens and other foreign invaders. They are derived from hematopoietic stem cells in the bone marrow and circulate throughout the body via the blood circulation system. White blood cells can be divided into five different types by their physical and functional characteristics. These include neutrophils, eosinophils, basophils, lymphocytes, and monocytes (Janeway, 2001).

Neutrophils are the most abundant white blood cells in human blood and account for about 40-65 % of all leukocytes. They form the first line of defence against bacterial and fungal infections by migrating through blood vessels into inflammatory sites and then eliminate the pathogens (Kolaczkowska and Kubes, 2013). Neutrophils possess a multi-lobe nucleus and their cytoplasm contains different types of granules containing essential proteins and enzymes that have different functions. These functions include killing ingested pathogens and digesting basement membranes in order to allow neutrophil migration from the circulation into sites of infection or inflammation (Segal, 2005).

The lifespan of neutrophils is normally shorter than other types of white blood cells, with an estimated life span around 8-20 h, but their life span can be prolonged during inflammation when they migrate from the blood circulation to infected tissue (Pillay et al., 2010). In an absence of cytokines and other pro-inflammatory agents, neutrophil undergo apoptosis before being removed by macrophages. This is to prevent them from releasing their cytotoxic contents to damage surrounded tissues (Savill et al., 1989). Neutrophil apoptosis can be regulated via several mechanisms such as Ca^{2+} influx shutdown, release of cytochrome c from mitochondria, and exposure of phosphatidylserine on the outer leaflet of the plasma membrane (Akgul et al., 2001, Ayub and Hallett, 2004).

Other leukocytes include mononuclear phagocytes (that can differentiate into macrophages) and lymphocytes. The latter cells can be distinguished by their round nucleus with relatively small volumes of cytoplasm (Janeway, 2001). Lymphocytes can be divided into T cells, B cells, and natural killer (NK) cells. These cells have different roles in immune function: T cells bind to antigenic peptides presented on major histocompatibility molecules (MHC) class II of antigen presenting cells and then produce cytokines that coordinate with immune response to kill infected cells (Josefowicz et al., 2012). The second group is B cells. They play a major role in antibody production. These antibodies bind to pathogens and then enhance pathogen destruction (Mauri and Bosma, 2012). Lastly, NK cells kill infected cells that do not display MHC molecule especially in the situation of viral infection and cancer (Vivier et al., 2008).

Monocytes can be distinguish from their relatively large size with unilobar nucleus (Janeway, 2001). They can migrate from vasculature into tissue and become macrophages to remove cell debris and attack pathogens. Apart from their phagocytic function, they also present pathogens to T cells to achieve additional immune response (Saha and Geissmann, 2011).

Apoptosis plays a key role in lymphocyte homeostasis. Enhance lymphocyte apoptosis leads to immunodeficiency diseases. Also, decrease in lymphocyte apoptosis resulted in autoimmune diseases and lymphoma (Rathmell and Thompson, 2002). Apoptosis of these cells is regulated by various signal transductions. These includes death receptor Fas (CD95/APO-1) and Bcl-2 family proteins (Xu and Shi, 2007).

In conclusion, white blood cells are major parts of cells in blood circulation together with red blood cells and platelets. They are functionally important in immune system and their apoptosis is highly regulated via several pathways. Therefore, effects of purvalanol A on white blood cells apoptosis are investigated in this Chapter.

From all above, neutrophils and PBMCs represent most of population of white blood cells. For this reason, these cells were used as validate models of actual circulating white blood cells.

The aims of the work described in this Chapter were:

i) To determine the effects of purvalanol A on apoptosis of neutrophils and PBMCs.

ii) To measure the expression of Bcl-2 family members in these white blood cells and to determine how their protein levels were affected by purvalanol A.

iii) To investigate the effects of purvalanol A on the activation status of intracellular signalling systems.

6.2 Methods

Imatinib and purvalanol A were used at 10 µM and 30 µM, respectively, which was shown in previous Chapters to induce maximal effects on the growth of CML cell lines. GM-CSF was used at 5 ng/ml as this concentration was previously shown to increase neutrophil survival in (Derouet et al., 2004, Derouet et al., 2006)

Purvalanol A and imatinib were dissolved in DMSO, and so control experiments contained equivalent amounts of this solvent (0.1-0.3 %, v/v). These concentrations did not have any measured effect of any of the cell parameters analysed on neutrophils and PBMCs.

6.3 Results

The purpose of the experiments in this Chapter was to measure the effects of purvalanol A on apoptosis of white blood cells using the Annexin V and PI assay and cell morphology, while the levels of expression of anti-apoptotic proteins, including Mcl-1, Bcl-2, and Bcl-X_L were measured by western blotting. The effects of purvalanol A on the activation status of several intracellular signalling systems, including Erk, STAT3, Akt, and p38 were also investigated. Also, the effects of BIRB796 was determined.

6.3.1 Effects of imatinib and purvalanol A on primary leukocyte apoptosis

Firstly, the effects of purvalanol A on apoptosis of white blood cells were determined by measuring Annexin V binding and PI staining by flow cytometry (see Materials and Methods). Neutrophils and PBMCs were isolated from three healthy donors (n=3) and incubated with AB serum with or without imatinib and purvalanol A to investigate whether cell apoptosis was affected by these agents. Neutrophils and PBMCs were incubated *in vitro* with 10 μM imatinib, 30 μM purvalanol A, and both together in order to compare the effects of these concentrations of drugs used with CML cells in the previous Chapter. Cells were incubated for a total of 18 h and the percentage of cell apoptosis was compared between untreated and treated cells using flow cytometry.

There was no significant difference in the percentage of neutrophil apoptosis (both early (annexin V positive) and late (PI staining) apoptosis) between untreated control, imatinib, purvalanol A treated, and imatinib plus purvalanol A treated cells, after 18 h of incubation (Figure 6.1). There was a trend for increased apoptosis in neutrophils exposed to imatinib and purvalanol A, but this did not reach statistical significance ($p>0.05$, $n=3$) when compared to the untreated control.

Imatinib, purvalanol A, and combination treatment did not induce significant levels of PBMC apoptosis compared to untreated controls (Figure 6.1). Similarly to neutrophils, there was a trend for increased apoptosis when cells were treated with these agents, but this also did not reach statistical significance ($p>0.05$, $n=3$). The percentage of PBMC apoptosis was relatively lower when compared to neutrophil incubation under the same conditions. However, the percentage of early apoptosis of untreated neutrophils was relatively higher than untreated PBMCs (64.2 ± 5.3 % and 10.2 ± 0.2 %, respectively).

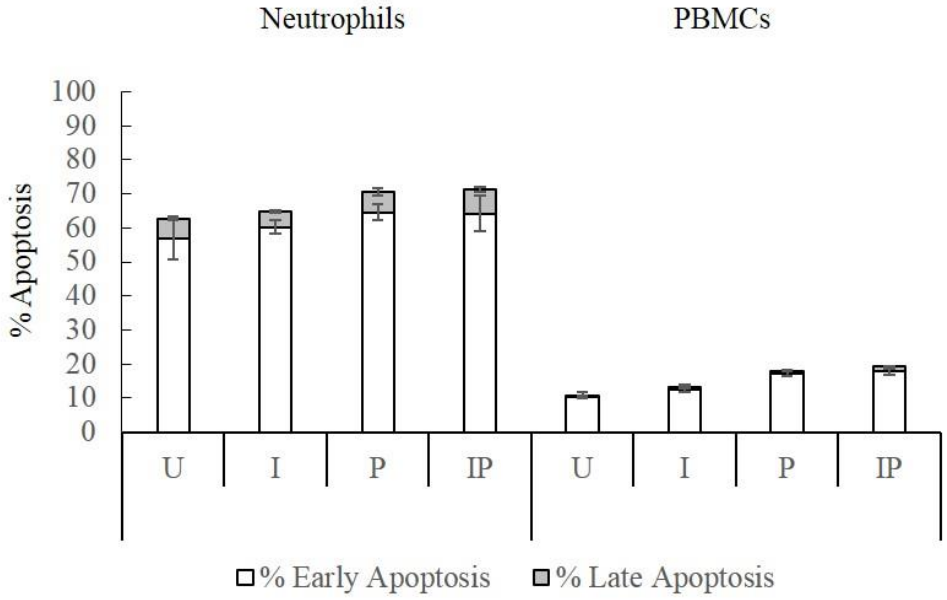

Figure 6.1 Effects of imatinib and purvalanol A on primary leukocyte apoptosis. Neutrophils and PBMCs were isolated from three healthy donors and incubated for 18 h in the absence (U) and presence (I), (P) and (IP) of imatinib (10 μM), Purvalanol A (30 μM), and the combination of imatinib (10 μM) plus Purvalanol A (30 μM). Apoptosis was assessed by flow cytometry using annexin V and PI. Data represent mean ± SEM.

Therefore, these results show that there was a trend for purvalanol A to induce apoptosis in white blood cells, but this did not reach statistical significance. However, high levels of neutrophil apoptosis were detected in the untreated control after 18 h incubation (>50 % of cells underwent early apoptosis). This suggests that 18 h incubation may be too long to measure effects of purvalanol A on apoptosis of neutrophils, which have a short half-life. Therefore, the effects of this drug on neutrophil apoptosis over shorter incubation times (0-6 h) were determined.

6.3.2 Effects of purvalanol A on primary leukocyte apoptosis over short incubation times

Both neutrophils and PBMCs were incubated with 30 μM purvalanol A for 0, 2, 4, and 6 h before measuring apoptosis by Annexin V and PI staining. In neutrophils, there was a trend for increased apoptosis over time after purvalanol A treatment. Purvalanol A significantly induced both early and late apoptosis ($p \leq 0.05$, n=3) after 6 h of incubation (untreated cells at 6 h; early 9.5 ± 0.5 % and late 0.3 ± 0.1 %: purvalanol A treated cells at 6 h; early 33.8 ± 4.2 % and late 0.9 ± 0.1 %, Figure 6.2A).

In contrast, purvalanol A induced only low levels of PBMC apoptosis (<15% of cells that underwent early apoptosis after 6 h incubation, Figure 6.2B).

Another parameter which can be used to detect apoptosis is cell morphology. Cell samples were stained and visualised by light microscopy. Neutrophils and PBMCs were incubated in the absence (untreated control) and presence of purvalanol A for total 6 h. These results showed that purvalanol A caused no significant increase in neutrophil and PBMC cell morphology by this incubation time. After purvalanol A treatment, some neutrophils were misshaped and showed condensed chromatin after 6 h incubation (cells in Figure 6.3, particularly inset at high magnification). However, cells with this morphology were also found in untreated neutrophils after the same (6 h) incubation time.

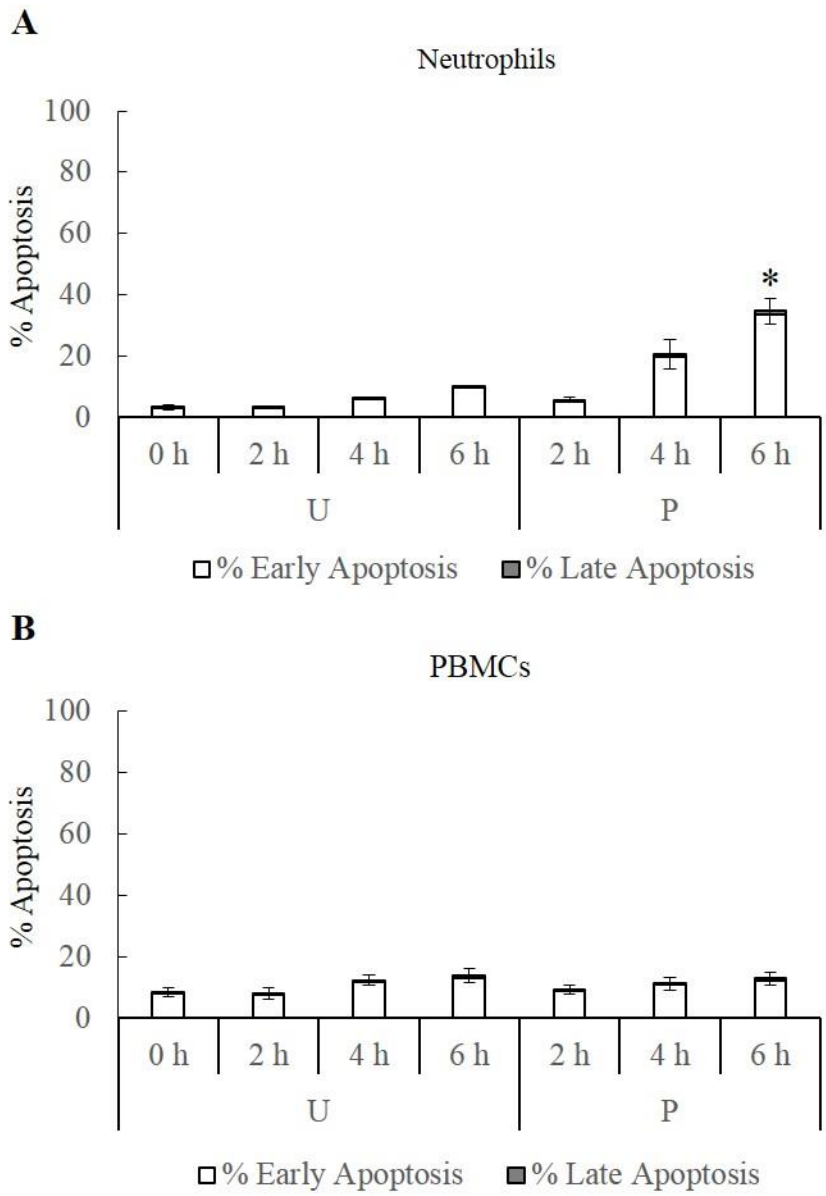

Figure 6.2 Effects of short term purvalanol A incubation on primary leukocyte apoptosis. Neutrophils, and PBMCs were isolated from three healthy donors and incubated for 0-6 h in the absence (U) and presence (P) of 30 μM purvalanol A. Apoptosis was assessed by flow cytometry using annexin V and PI. Data are shown as mean (±SEM, n=3) *=p≤0.05 (paired two-tailed student's t-test compared to untreated control at the same time point)

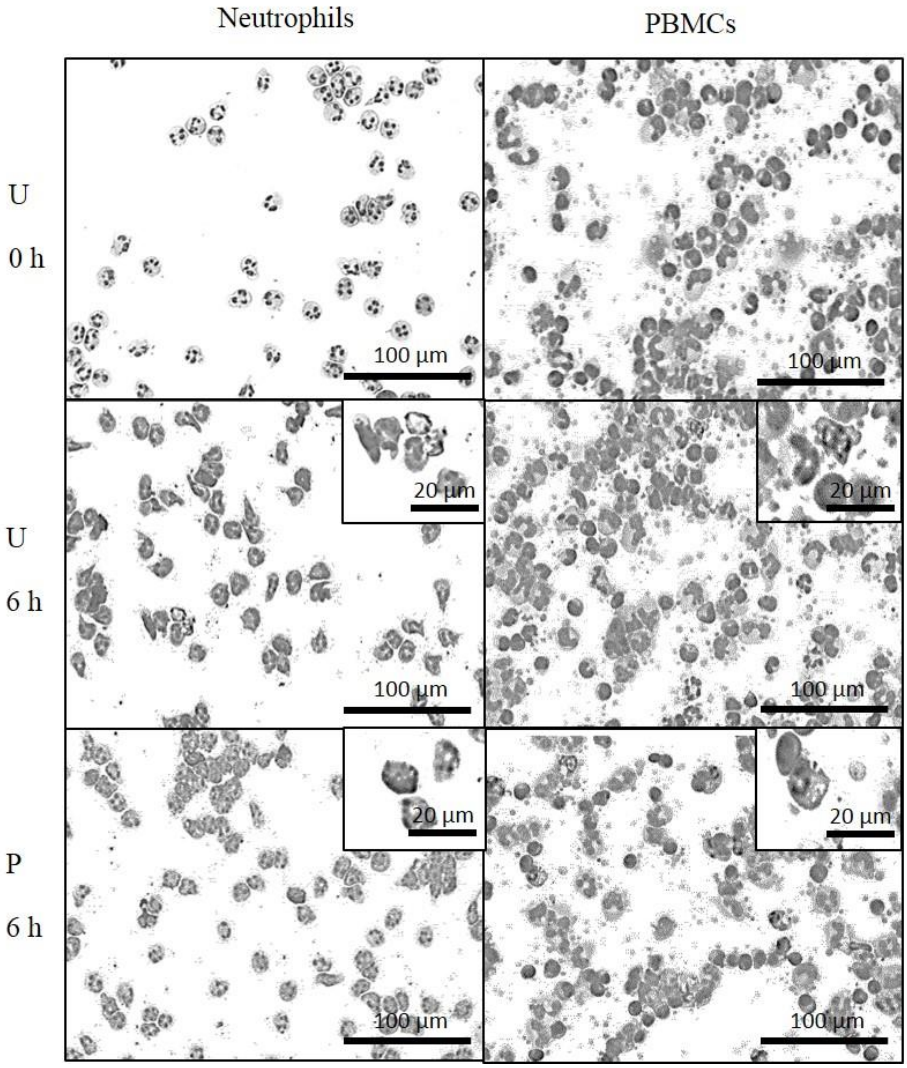

Figure 6.3 Effect of purvalanol A on primary leukocyte morphology. Both neutrophils and PBMCs were incubated for 6 h in the absence (U) and presence (P) of purvalanol A (30 µM). Cell morphology was observed by light microscopy after staining of cytospins (see Materials and Methods).

In the same way as with neutrophils, some purvalanol A treated PBMCs were misshaped and showed condensed chromatin after 6 h of incubation. However, these apoptotic appearances were also found in untreated PBMCs at the same (6 h) incubation time (Figure 6.3).

6.3.3 Effects of purvalanol A on Mcl-1 expression in primary leukocytes

The anti-apoptotic protein, Mcl-1, plays a key role in survival of primary immune cells and apoptosis, especially in neutrophils (Moulding et al., 2001). Neutrophils and PBMCs were therefore incubated in the absence (untreated control) and presence of 30 µM purvalanol A up to 6 h and protein lysates were collected at 0, 2, 4, and 6 h. The expression of Mcl-1 in these cells were determined by western blotting (normalised to actin).

Mcl-1 expression in neutrophils incubated with purvalanol A was significantly decreased after 4 h of incubation (65.3 ± 4.2 %) compared to untreated control at the same time (4 h) (90.9 ± 1 %), ($n=3$, $p \leq 0.05$), (Figure 6.4). Similar to neutrophils, Mcl-1 expression in PBMCs incubated with purvalanol A was also decreased after 6 h of incubation (68.2 ± 4.3 %) compared to untreated control at 6 h (93.6 ± 3.2 %), ($n=3$, $p \leq 0.05$), (Figure 6.5).

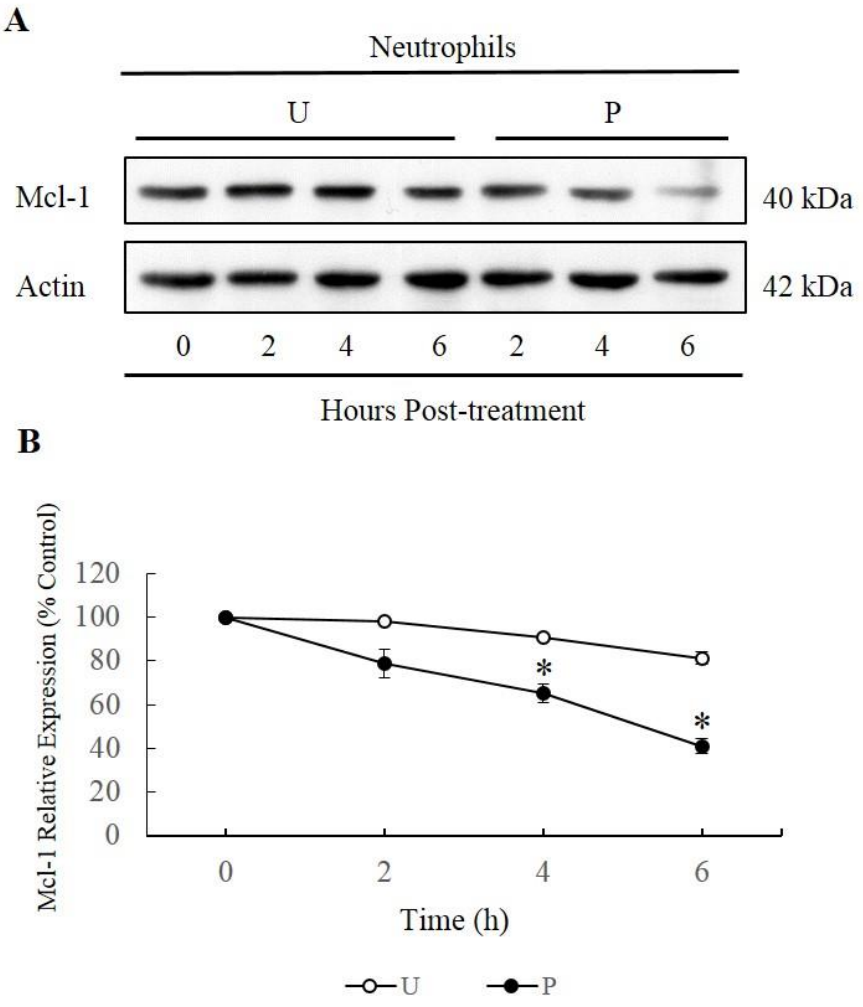

Figure 6.4 Effects of purvalanol A on Mcl-1 expression in neutrophils. Cells were incubated in the absence (U ○) or presence of 30 μM purvalanol A (P ●) for 2, 4 and 6 h before preparation for Western blotting for Mcl-1 (40 kDa) and β-actin (42 kDa), quantified by densitometry. **(A)** shows typical western blots, while **(B)** shows densitometry analysis, expressed as a % of untreated control, untreated samples at time zero (±SEM, n=3), * = $p \leq 0.05$, (paired two-tailed student's t-test). Western blot shown is representative from n=3 experiments.

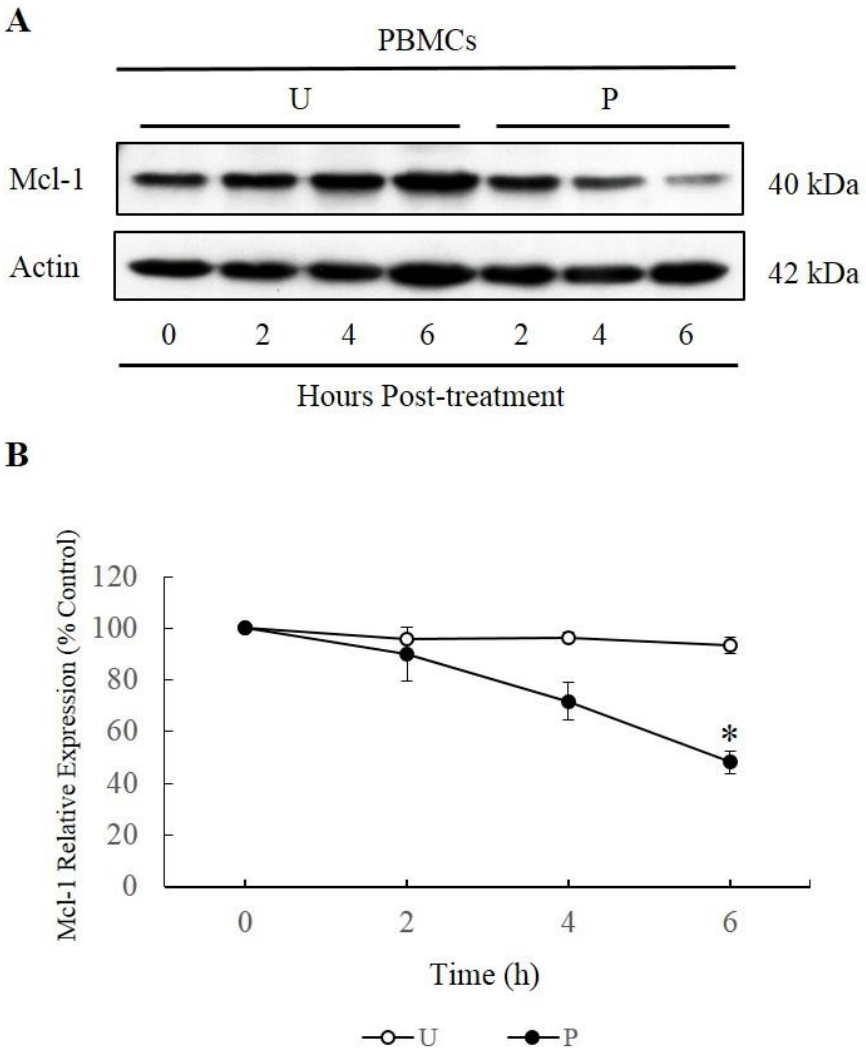

Figure 6.5 Effects of purvalanol A on Mcl-1 expression in PBMCs. Cells were incubated in the absence (U ○) or presence of 30 μM purvalanol A (P ●) for 2, 4 and 6 h before preparation for Western blotting for Mcl-1 (40 kDa) and β-actin (42 kDa), quantified by densitometry. **(A)** shows typical western blots, while **(B)** shows densitometry analysis, expressed as a % of untreated control, untreated samples at time zero (±SEM, n=3), * = $p \leq 0.05$, (paired two-tailed student's t-test). Western blot shown is representative from n=3 experiments.

6.3.4 Effects of purvalanol A on other anti-apoptotic protein expression in PBMCs

Although, purvalanol A had a little effect on PBMCs apoptosis, it did significantly decrease Mcl-1 expression in PBMCs after 6 h incubation ($p \leq 0.05$, n=3). Thus, the effects of purvalanol A on other anti-apoptotic proteins were determined to investigate effects on other anti-apoptotic proteins that would protect against cell death. PBMCs were incubated with and without 30 µM purvalanol A for up to 6 h and protein lysates were collected at 0, 2, 4, and 6 h. The expression of Bcl-2 and Bcl-X_L in these cells were determined by western blotting (normalised to actin).

6.3.4.1 The effects of purvalanol A on Bcl-2 expression in PBMCs

In contrast to Mcl-1, Bcl-2 expression in purvalanol A treated PBMCs continued to be increase over time. Bcl-2 expression in purvalanol A treated PBMCs was significantly increased after 6 h incubation (at 126.2 ± 3.7 %) compared to untreated control at the same time (at 94.6 ± 2.1 %), ($p \leq 0.05$, n=3). (Figure 6.6).

6.3.4.2 The effects of purvalanol A on Bcl-X_L expression in PBMCs

Bcl-X_L expression in purvalanol A treated PBMCs remained at approxmately the same level over time compared to untreated control (Figure 6.7).

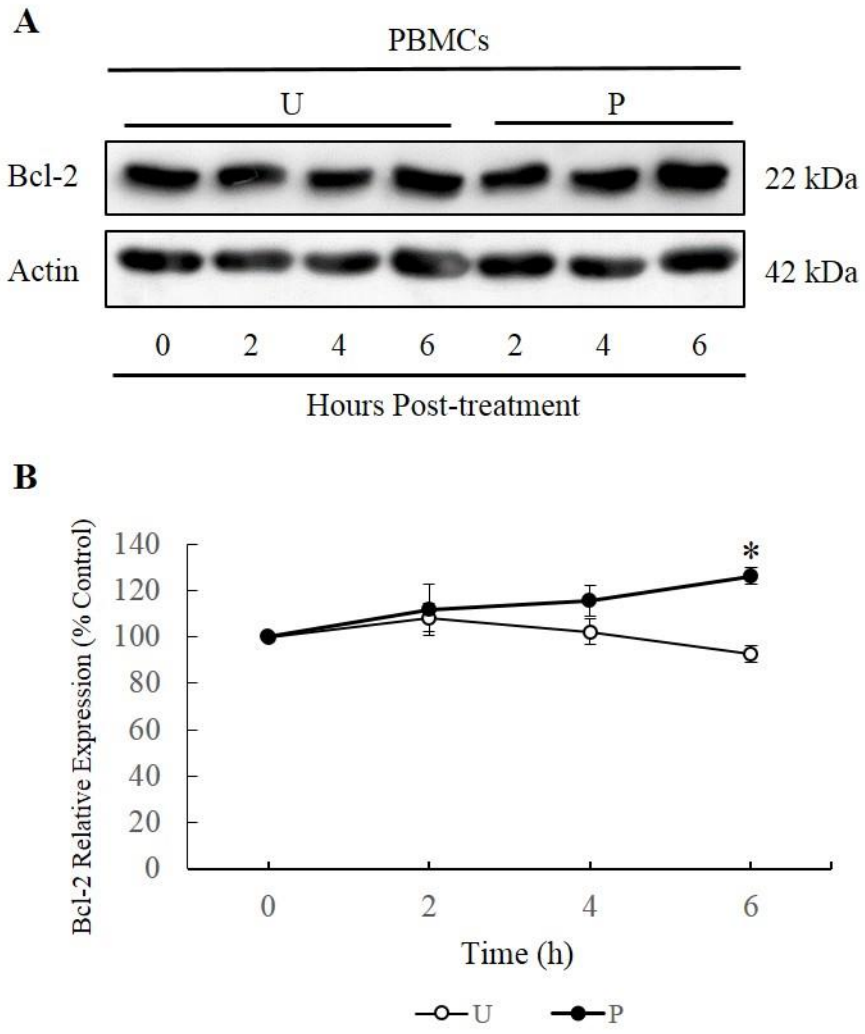

Figure 6.6 Effects of purvalanol A on Bcl-2 expression in PBMCs. Cells were incubated in the absence (U ○) or presence of 30 µM purvalanol A (P ●) for 2, 4 and 6 h before preparation for Western blotting for Bcl-2 (22 kDa) and β-actin (42 kDa), quantified by densitometry. **(A)** shows typical western blots, while **(B)** shows densitometry analysis, expressed as a % of untreated control, untreated samples at time zero (±SEM, n=3), * = p≤0.05, (paired two-tailed student's t-test). Western blot shown is representative from n=3 experiments.

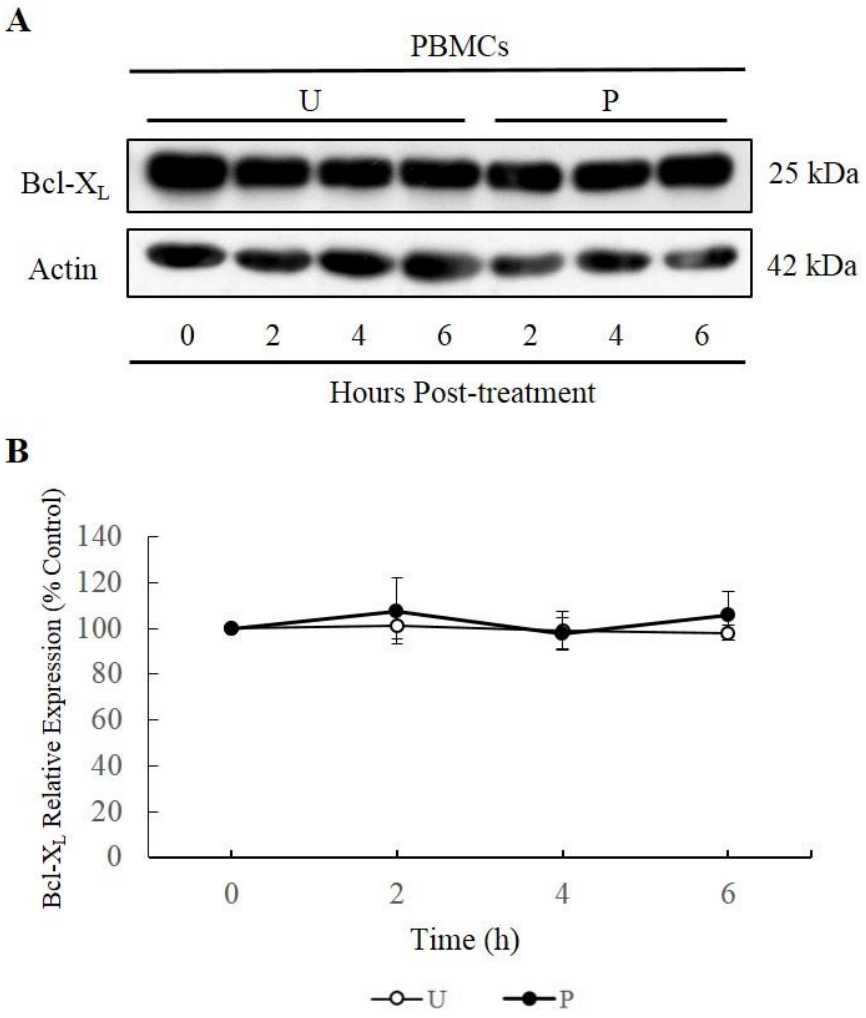

Figure 6.7 Effects of purvalanol A on Bcl-X_L expression in PBMCs. Cells were incubated in the absence (U ○) or presence of 30 µM purvalanol A (P ●) for 2, 4 and 6 h before preparation for Western blotting for Bcl-X_L (25 kDa) and β-actin (42 kDa), quantified by densitometry. **(A)** shows typical western blots, while **(B)** shows densitometry analysis, expressed as a % of untreated control, untreated samples at time zero (±SEM, n=3). Western blot shown is representative from n=3 experiments.

6.3.5 Effects of purvalanol A and GM-CSF on neutrophils.

As shown in previous results, purvalanol A significantly decreased Mcl-1 expression in neutrophils after 4 h incubation ($p \leq 0.05$, n=3). GM-CSF has been shown to delay neutrophil apoptosis and stabilize Mcl-1 protein levels and thus, the effects of purvalanol A were investigated in the absence and presence of GM-CSF.

6.3.5.1 Effects of purvalanol A and GM-CSF on neutrophil apoptosis.

Neutrophils were incubated with 30 μM purvalanol A in the absence and presence of 50ng/ml GM-CSF for 0, 2, 4, and 6 h before measuring apoptosis by Annexin V and PI binding. Figure 6.8 shows that GM-CSF decreased neutrophil apoptosis (% early and % late) induced by purvalanol A. When used as a single agent, purvalanol early and late apoptosis at 6 h was 33.8 ± 4.2 % and 0.9 ± 0.1 %, respectively, whereas in combination with GM-CSF, early and late apoptosis were 15.2 ± 4.9 % and 0.7 ± 0.2 %, respectively. ($p > 0.05$, n=3).

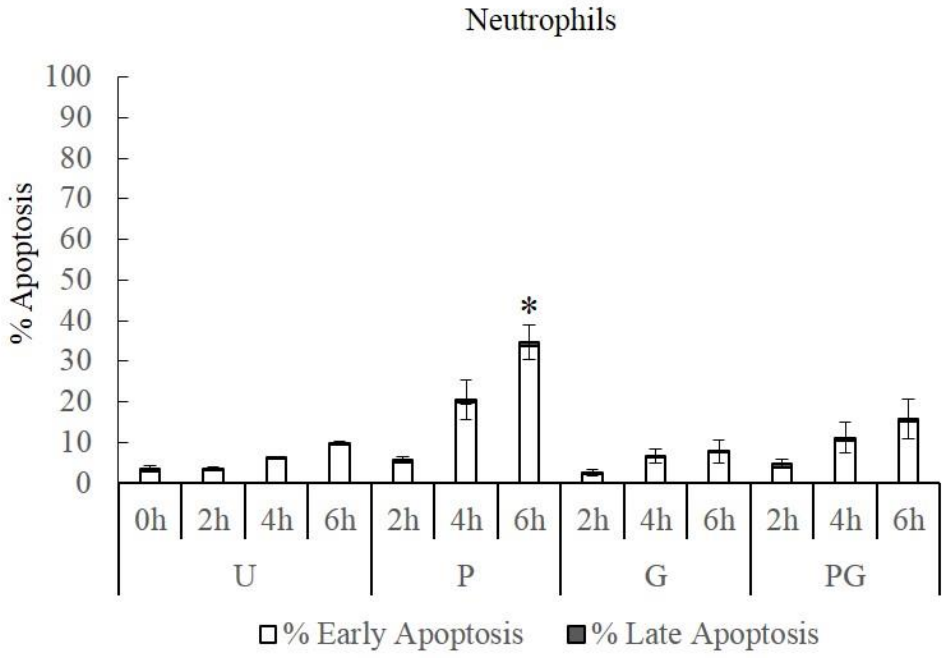

Figure 6.8 Effects of purvalanol A and GM-CSF on neutrophil apoptosis. Neutrophils were isolated from three healthy donors and incubated for 0-6 h in the absence (U) and presence of 30 μM purvalanol A (P), 50 ng/ml GM-CSF (G), and both together (PG). Apoptosis was assessed by flow cytometry using annexin V and PI. Data are shown as mean (±SEM, n=3), *=p≤0.05, (paired two-tailed student's t-test) compared to untreated controls at the same time point.

6.3.5.2 Effects of purvalanol A and GM-CSF on Mcl-1 expression in neutrophils.

Neutrophils were incubated as described in 6.3.6.1 and cell lysates were prepared for western blotting. Figure 6.9 confirms that purvalanol significantly decreased Mcl-1 expression in neutrophils after 4 h incubation (60.8 ± 10.2 %) compared to untreated control (at 101.1 ± 2.8 %), ($p \leq 0.05$, n=3). In addition, co-incubation of purvalanol A with GM-CSF increased Mcl-1 levels (at 67.8 ± 5.6 %) compared to purvalanol A treated cells (at 60.8 ± 10.2 % (4 h)). This suggests that the effect of GM-CSF slightly interfered with the effect of purvalanol A in decreasing Mcl-1 expression in neutrophils.

GM-CSF alone significantly increased Mcl-1 expression in neutrophils (at 123.8 ± 3.8 % at 2 h) compared to untreated controls (at 105 ± 23.2 % at 2 h), (n=3, $p \leq 0.01$). In addition, there was a significant difference between GM-CSF treated expression and after co-incubation with GM-CSF and purvalanol A at 4 and 6 h of incubation, ($p \leq 0.05$, n=3).

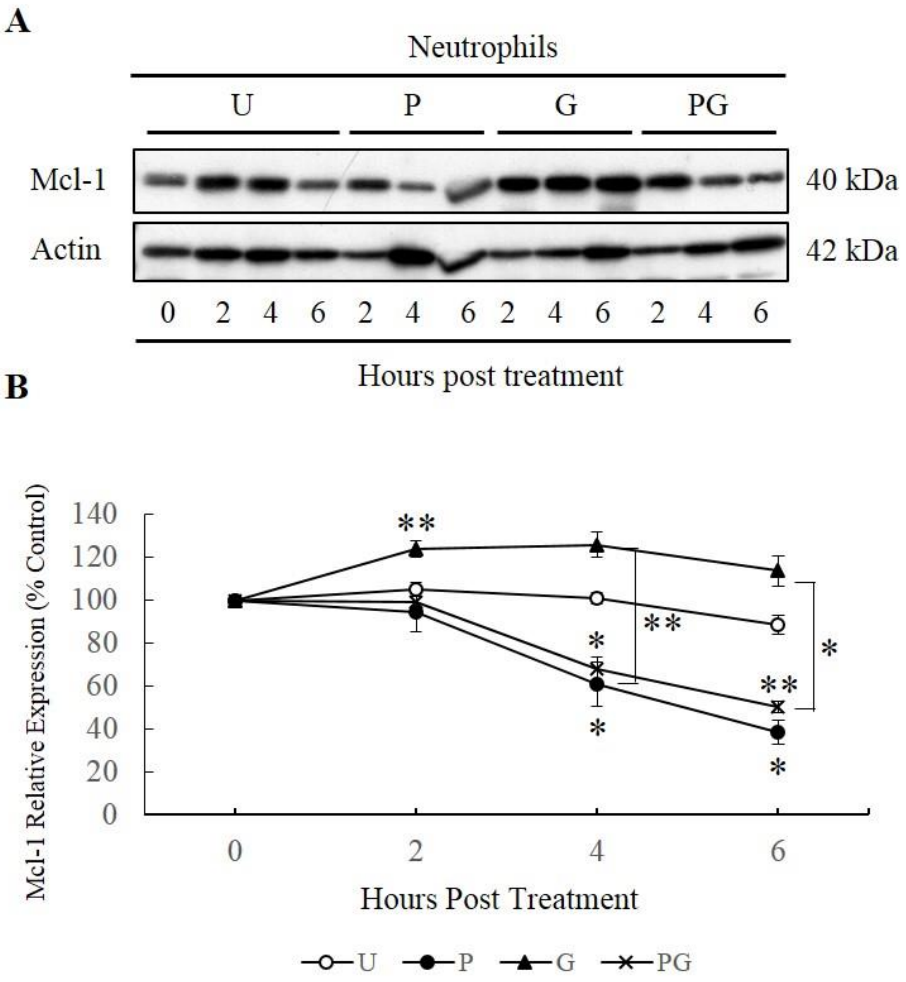

Figure 6.9 Effects of purvalanol A and GM-CSF on Mcl-1 expression in neutrophils. Cells were incubated in the absence (U ○) or presence of 30 μM purvalanol A (P ●), 50ng/ml GM-CSF (G ▲), and both (PG x) for 2, 4, and 6 h before preparation for Western blotting for Mcl-1 (40 kDa) and β-actin (42 kDa), quantified by densitometry. **(A)** shows typical western blots, while **(B)** shows densitometry analysis, expressed as a % of untreated control samples at time zero (±SEM, n=3), * = p≤0.05, **=p≤0.01, (One-way ANOVA followed by *Bonferroni post hoc* comparisons tests). Western blot shown is representative from n=3 experiments.

6.3.6 Effects of purvalanol A on half-life of Mcl-1 in neutrophils.

The following experiments were performed to determine if purvalanol A caused apoptosis via changes in the turnover rate of Mcl-1 in neutrophils, as has previously been reported in this thesis for CML cell lines. Neutrophils were incubated with cycloheximide (CHX) for 10 min before incubating with purvalanol A for 1, 2, 3, and 4 h in the absence and presence of 30 μM of purvalanol A, and protein extracts were prepared. Levels of Mcl-1 protein in these cells were determined by western blotting.

This result shows that when *de novo* protein synthesis was blocked by cycloheximide, Mcl-1 was constitutively degraded and this turnover was accelerated in the presence of purvalanol A (Figure 6.10). The half-life of Mcl-1 in untreated, control neutrophils was approximately 3 h and 30 min, whilst in the presence of purvalanol A, this had decreased to 2 h and 30 min ($p \geq 0.05$, n=3).

Mcl-1 levels were significantly lower after 6 h after CHX treatment in the presence of purvalanol A incubation (20.1 ± 5.3 %) compared to treatment with CHX alone at the same time (42.1 ± 2.7 %), ($p \leq 0.05$, n=3).

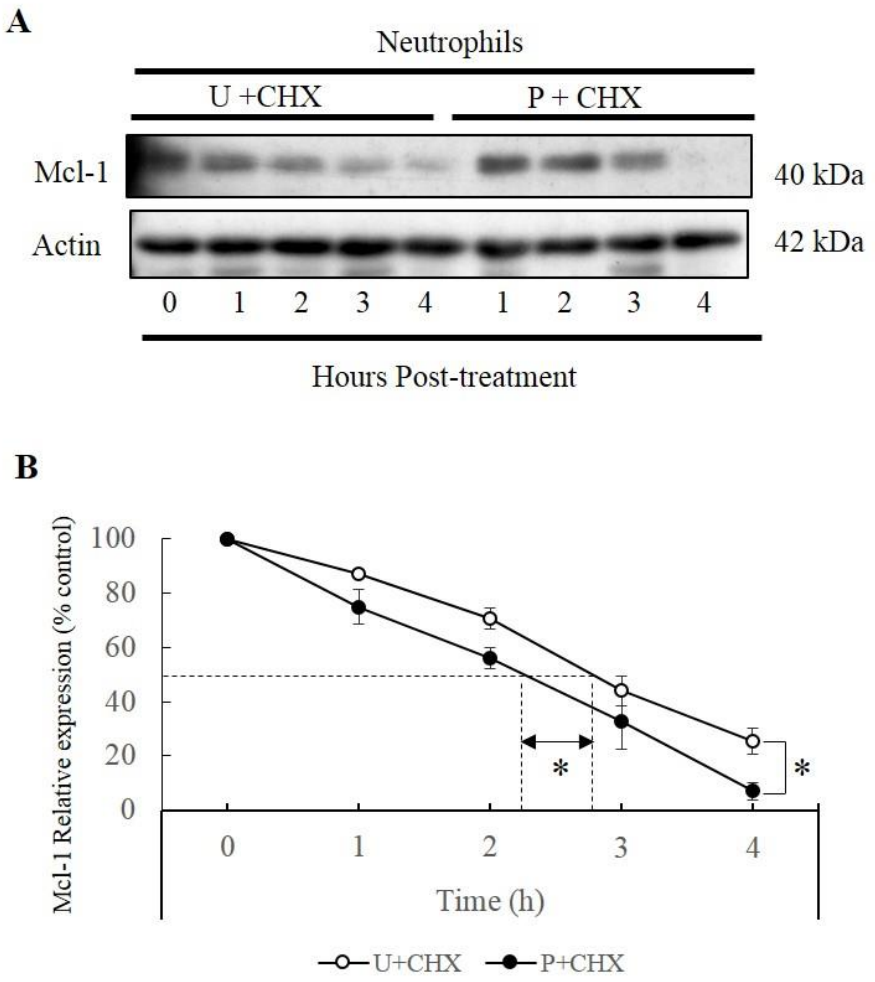

Figure 6.10 Effect of purvalanol A on the half-life of Mcl-1 in neutrophils. Neutrophils were pre-incubated for 10 min with 10 g/mL cycloheximide (CHX) prior to 0, 1, 2, 3, and 4 h incubation in the absence (U), or presence of 30 μM purvalanol A (P). Cell samples were collected at the times indicated and prepared for Western blotting for the levels of Mcl-1 (40 kDa). **(A)** shows typical western blots, while **(B)** shows densitometry analysis, expressed as a % of untreated control samples at time zero (±SEM, n=3), * = $p \leq 0.05$, (paired two-tailed student's t-test). Western blot shown is representative from n=3 experiments.

6.3.7 Effects of purvalanol A and GM-CSF on intracellular signalling pathways in neutrophils

The effects of purvalanol A and GM-CSF on the activation status of several intracellular signalling systems were subsequently investigated. Neutrophils were incubated with 30 µM purvalanol A and/or 50 ng/ml GM-CSF for 15, 30 min and 1 h before the cell lysates were prepared and western analysis was performed to determine the effects of purvalanol A and GM-CSF on neutrophil signalling pathways. Erk, STAT3, Akt, and p38 signalling pathways were investigated in this study.

6.3.7.1 Erk signalling pathway

GM-CSF increased levels of pErk in neutrophils significantly after 15 min of incubation (1428.1 ± 233.4 %) compared to untreated control at the same time (99.9 ± 0.3 %), ($p \leq 0.05$, n=3). In contrast, purvalanol A treatment resulted in a slight increase in pErk levels after 15 min of incubation and then this effect continued to decrease after 30 min and 1 h of incubation. The co-incubation of purvalanol A and GM-CSF resulted in a partial increase in pErk levels after 15 min incubation (n=3, $p > 0.05$).

There was a significant difference between the combination treatment (504.2 ± 102.2 %) and GM-CSF treated cells (934.3± 71 %) after 1 h of incubation, ($p \leq 0.05$, n=3), (Figure 6.11).

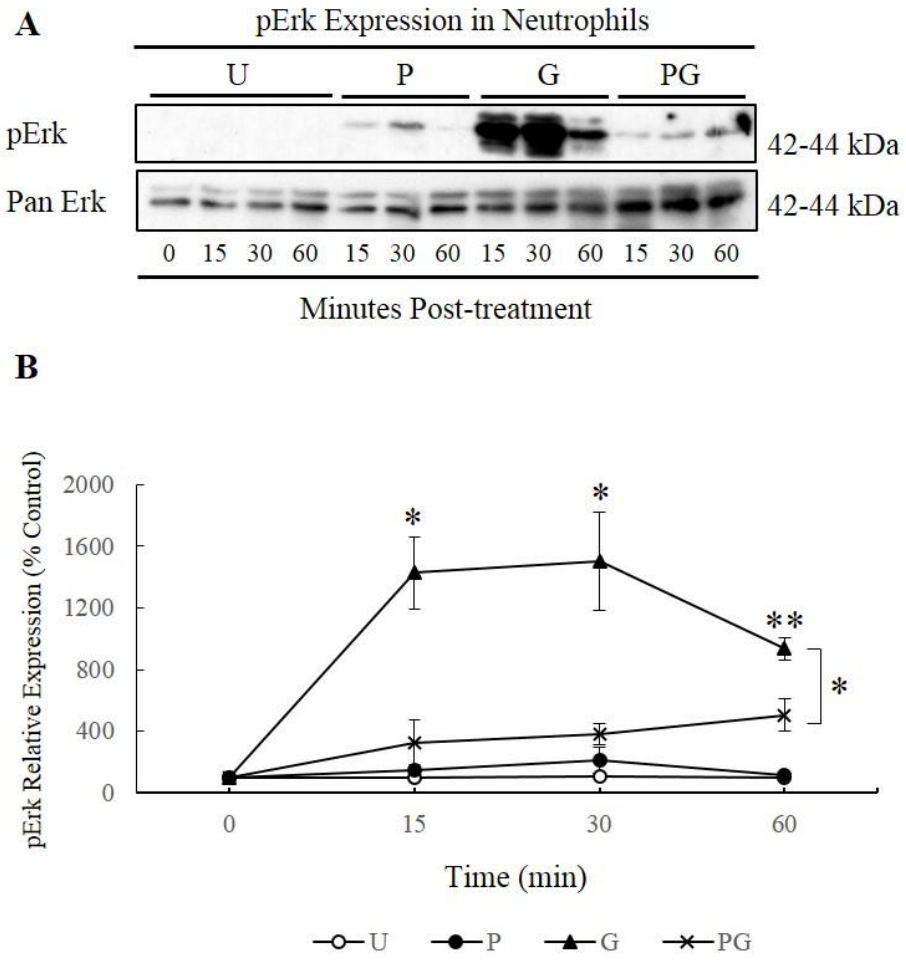

Figure 6.11 Effects of purvalanol A and GM-CSF on Erk activation in neutrophils. Cells were incubated in the absence (U ○) or presence of 30 μM purvalanol A (P ●), 5 ng/ml GM-CSF (G ▲), and both (PG x) for 15, 30 min and 1 h before preparation for Western blotting for pErk and Pan Erk (42-44 kDa), quantified by densitometry. **(A)** shows typical western blots, while **(B)** shows densitometry analysis, expressed as a % of untreated control, untreated samples at time zero (±SEM, n=3), * = $p \leq 0.05$, **=$p \leq 0.01$ (One-way ANOVA followed by *Bonferroni post hoc* comparisons tests). Western blot shown is representative from n=3 experiments.

6.3.7.2 STAT3 signalling pathway

Similar to Erk signalling pathway, GM-CSF increased levels of pSTAT3 significantly after 15 min of incubation (1492.3 ± 124.4 %) compared to untreated controls at the same time points (103.2 ± 0.6 %), ($p \leq 0.01$, n=3). In contrast, both purvalanol A and co-incubation with GM-SCF and purvalanol A resulted in no significant change in pSTAT3 levels at any time point measured, ($p > 0.05$, n=3), (Figure 6.12), indicating that purvalanol A abrogated the stimulatory effect of GM-CSF on STAT3 activation.

6.3.7.3 Akt signalling pathway

Purvalanol A and GM-CSF treatment both increased pAkt levels significantly in neutrophils, ($p \leq 0.01$ and $p \leq 0.001$, respectively, n=3). The level of phosphorylated Akt was increased by almost 7-10 times compared to untreated control after incubation for 15 min (Figure 6.13).

Similar to purvalanol and GM-CSF as a single treatment, the combination treatment of purvalanol A and GM-CSF also increased the levels of pAkt significantly after 15 min of incubation (1301.5 ± 272.5 %) compared to untreated control at the same time (at 113.4 ± 2.8 %), ($p \leq 0.05$, n=3).

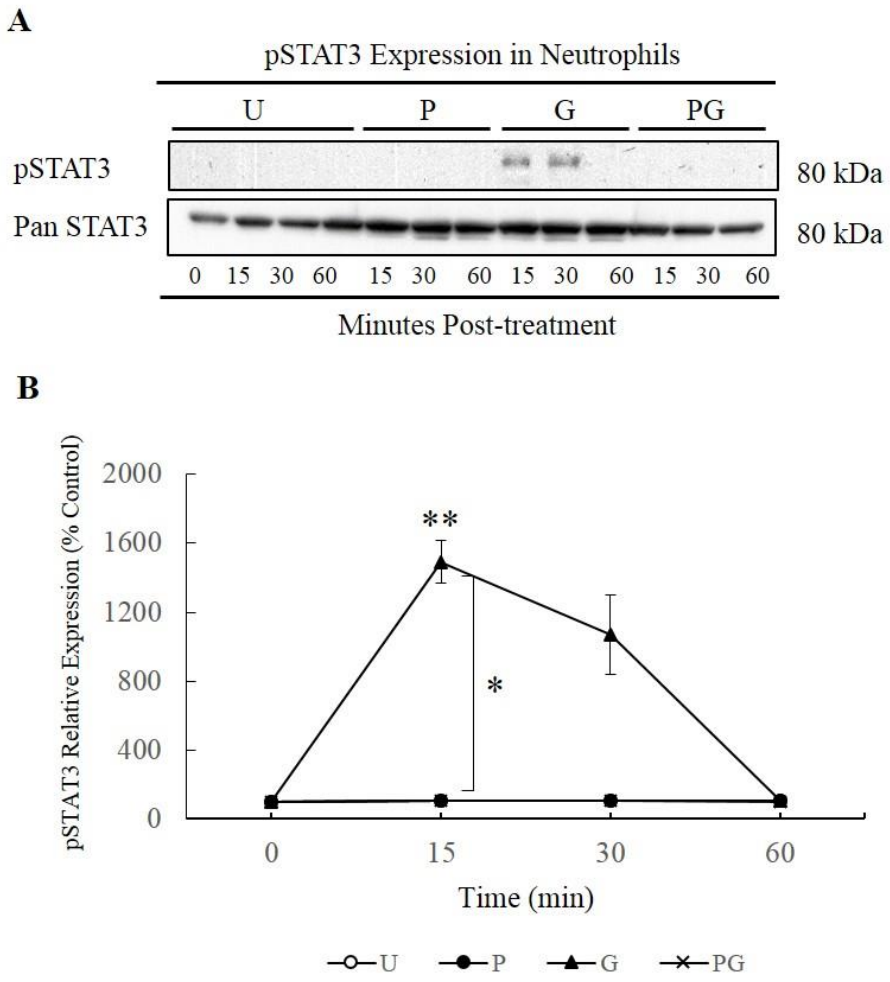

Figure 6.12 Effects of purvalanol A and GM-CSF on STAT3 activation in neutrophils. Cells were incubated in the absence (U ○) or presence of 30 μM purvalanol A (P ●), 5ng /ml GM-CSF (G ▲), and both (PG x) for 15, 30 min and 1 h before preparation for Western blotting for pSTAT3 and Pan STAT3 (80 kDa), quantified by densitometry. **(A)** shows typical western blots, while **(B)** shows densitometry analysis, expressed as a % of untreated control, untreated samples at time zero (±SEM, n=3), * = $p \leq 0.05$ (One-way ANOVA followed by *Bonferroni post hoc* comparisons tests). Western blot shown is representative from n=3 experiments.

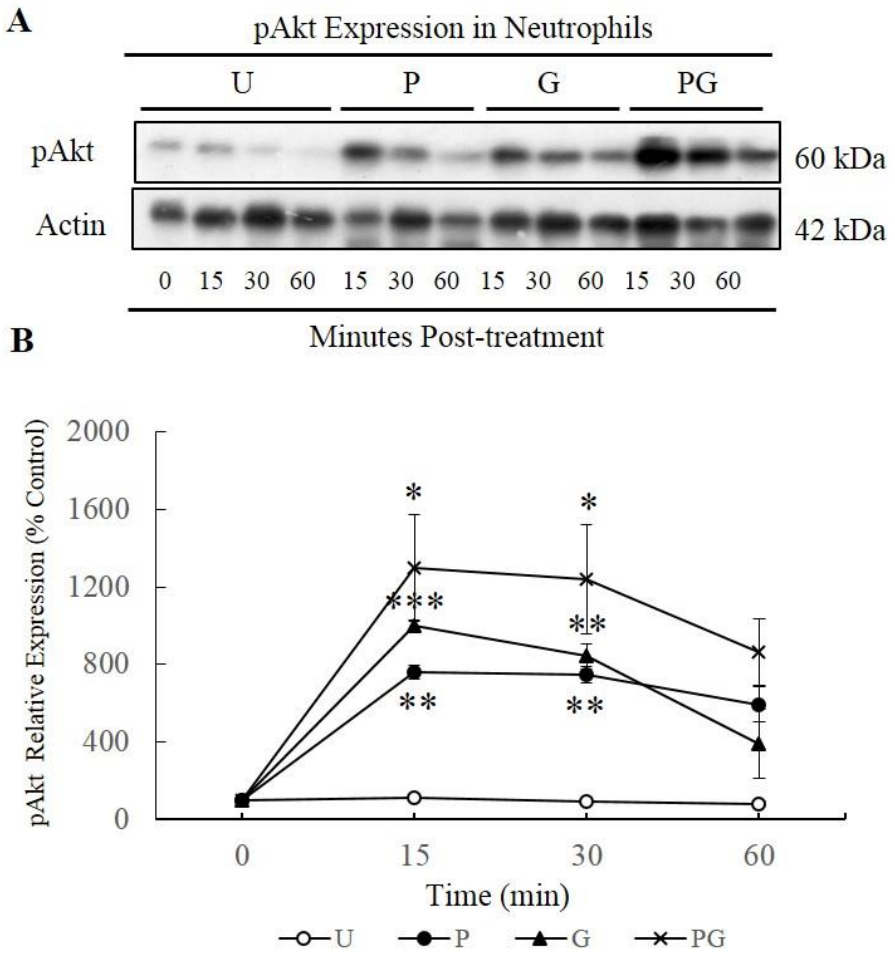

Figure 6.13 Effects of purvalanol A and GM-CSF on Akt activation in neutrophils. Cells were incubated in the absence (U ○) or presence of 30 µM purvalanol A (P ●), 5 ng/ml GM-CSF (G ▲), and both (PG x) for 15, 30 min and 1 h before preparation for Western blotting for pAkt (60 kDa) and Actin (42 kDa), quantified by densitometry. **(A)** shows typical western blots, while **(B)** shows densitometry analysis, expressed as a % of untreated control, untreated samples at time zero (±SEM, n=3), * = $p \leq 0.05$ (One-way ANOVA followed by *Bonferroni post hoc* comparisons tests). Western blot shown is representative from n=3 experiments.

6.3.7.4 p38 signalling pathway

In contrast to Erk, STAT3, and Akt signalling pathways, purvalanol A treatment resulted in a dramatic increased phosphorylated p38 in neutrophils. Purvalanol A significantly increased phosphorylated p38 after 15 min incubation (791.2 ± 87.7 %) compared to untreated controls at the same time (108.5 ± 34.2 %), ($p \leq 0.01$, n=3). Furthermore, co-incubation of purvalanol A and GM-CSF also increased the levels of Phospho-p38 significantly after 15 min incubation (828.6 ± 69.8 %) compared to untreated controls at the same time (108.5 ± 34.2 %), ($p \leq 0.01$, n=3). However, there was no significant change compared to the effect of purvalanol A as a single agent (Figure 6.14) that is the effects on p38 observed in the presence of GM-SCF and purvalanol A together were due to that observed by purvalanol A alone.

GM-CSF treatment resulted in an increase in the level of Phospho-p38 in neutrophils. However, this effect was lower than that observed in response to purvalanol A treatment. In contrast to Erk, STAT3, and Akt signalling pathways, GM-CSF treatment did not increase the level of Phospho-p38 significantly in neutrophils ($p > 0.05$, n=3).

6.3.8 Effects of purvalanol A and BIRB796 on expression of the Mcl-1 in neutrophils

Neutrophils were incubated in the absence (untreated control) and presence of 30 µM purvalanol A, 10 µM BIRB796, and both inhibitors together. Then, cell lysates were prepared for western blotting.

Figure 6.15 confirms that purvalanol decreased Mcl-1 expression in neutrophils In addition, co-incubation of purvalanol A with BIRB796 increased Mcl-1 levels significantly (at 62.2 ± 4.7 % (6 h)) compared to purvalanol A treated cells (at 28.4 ± 9.6 % (6 h)), ($p \leq 0.05$, n=3). This suggests that BIRB796 blocked the effect of purvalanol A in decreasing Mcl-1 expression in neutrophils.

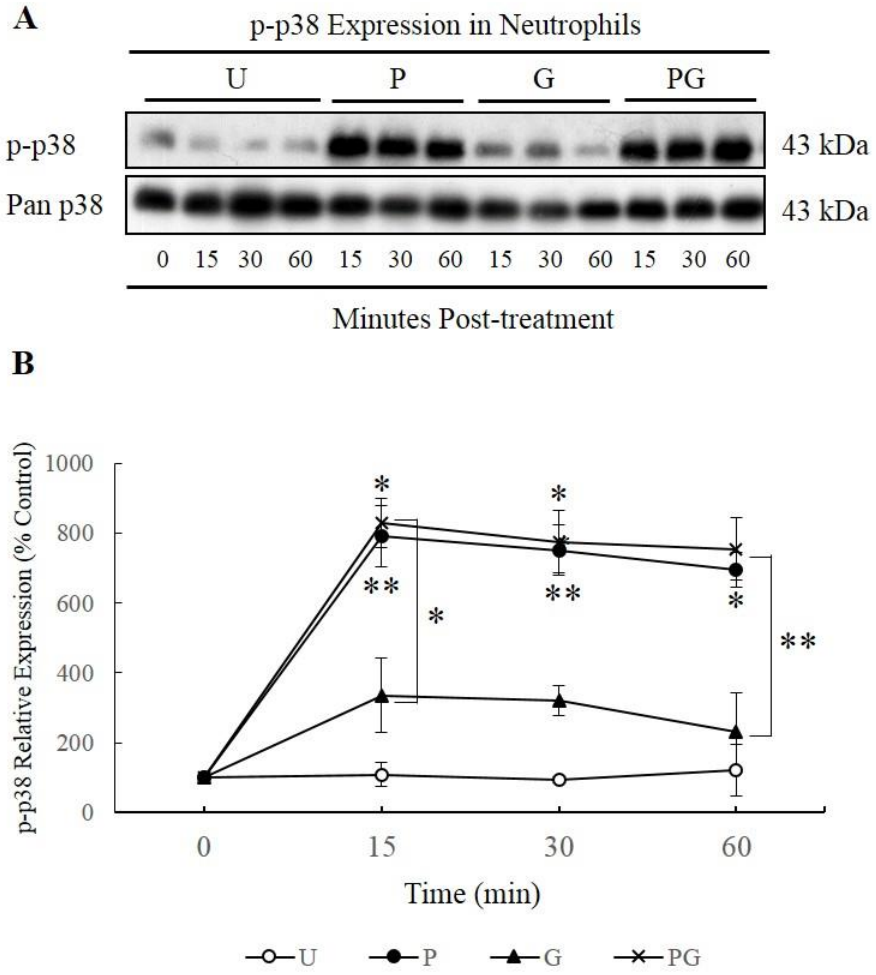

Figure 6.14 Effects of purvalanol A and GM-CSF on p38 activation in neutrophils. Cells were incubated in the absence (U ○) or presence of 30 μM purvalanol A (P ●), 5 ng/ml GM-CSF (G ▲), and both (PG x) for 15, 30 min and 1 h before preparation for Western blotting for Phospho-p38 and Pan p38 (43 kDa), quantified by densitometry. **(A)** shows typical western blots, while **(B)** shows densitometry analysis, expressed as a % of untreated control samples at time zero (±SEM, n=3), * = p≤0.05 (One-way ANOVA followed by *Bonferroni post hoc* comparisons tests). Western blot shown is representative from n=3 experiments.

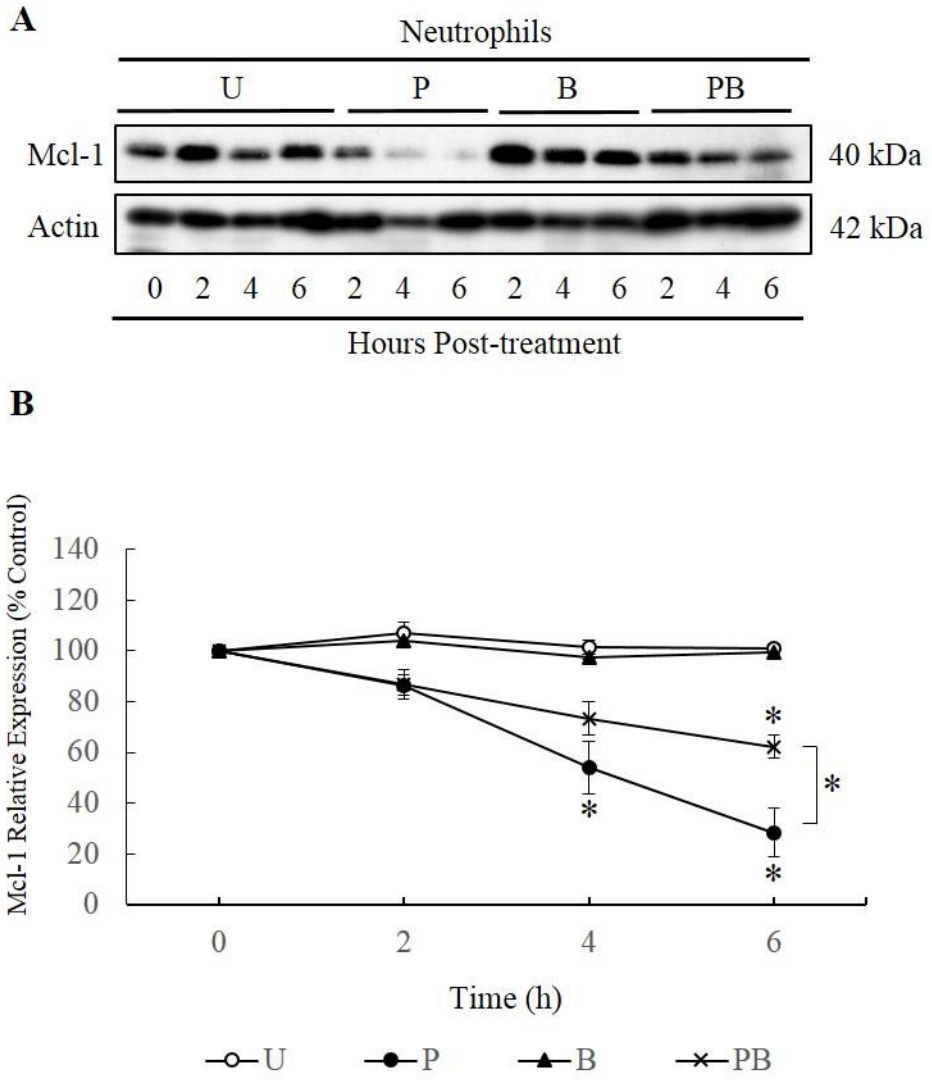

Figure 6.15 Effects of purvalanol A and BIRB796 on Mcl-1 expression in neutrophils. Cells were incubated in the absence (U ○) or presence of 30 μM purvalanol A (P ●), 10 μM BIRB796 (B ▲), and both (PB x) for 2, 4, and 6 h before preparation for Western blotting for Mcl-1 (40 kDa) and β-actin (42 kDa), quantified by densitometry. **(A)** shows typical western blots, while **(B)** shows densitometry analysis, expressed as a % of untreated control samples at time zero (±SEM, n=3), * = $p \leq 0.05$ (One-way ANOVA followed by *Bonferroni post hoc* comparisons tests). Western blot shown is representative from n=3 experiments.

6.4 Discussion and Conclusions

The effects of purvalanol A on neutrophils and PBMCs were investigated in this Chapter. This was to determine if this inhibitor could induce apoptosis in normal white blood cells as well as CML cell lines. This is an important factor to consider if purvalanol A, or compounds like it, could ever be used therapeutically to treat CML or other diseases.

The effects of purvalanol A on neutrophil and PBMC cell apoptosis were first investigated using Annexin V and PI binding. Both cell types were initially incubated with 10 µM imatinib and/or 30 µM purvalanol A for 18 h. However, more than 50% of the untreated neutrophil population underwent apoptosis after 18 h incubation. This confirms that neutrophils have a short half-life (around 8-20 h) (Pillay et al., 2010) but made it difficult to determine if purvalanol A rapidly induced neutrophil apoptosis. Therefore, subsequent experiments were performed using 0-6 h incubation time.

At 6 h incubation, purvalanol A significantly induced apoptosis in neutrophils, but not in PBMCs. As a consequence, the effects of purvalanol A on Mcl-1 expression were investigated by western blotting. This part of the study showed that purvalanol A also decreased Mcl-1 expression significantly in both neutrophils and PBMCs after 4 and 6 h incubation, respectively. These findings support previous studies which showed that other CDK inhibitors, such as roscovitine, induced apoptosis in neutrophils by decreasing Mcl-1 protein expression (Rossi et al., 2006). Also, it has been reported that purvalanol A inhibited leukocyte adhesion and migration from the blood circulation to tissue during infections (Liu et al., 2008).

The effects of purvalanol A on the expression of other anti-apoptotic proteins of the Bcl-2 family (Bcl-2 and Bcl-X_L) in PBMCs were also investigated in order to determine if changes in the expression levels of these proteins may explain the survival of these cells, even though the drug induced a decrease in Mcl-1 expression. Purvalanol A was found to increase the level of Bcl-2 expression in PBMCs significantly after 6 h incubation, while the levels of Bcl-X_L remained steady over this time

period. This result supports the concept that Bcl-2 plays a crucial role in an apoptosis of PBMCs, as other reports have shown that Bcl-2 is involved in PBMCs apoptosis from several chronic viral infections such as human immunodeficiency and hepatitis C virus (Boudet et al., 1996, Nakamoto et al., 2002). Also, PBMC numbers in mice with a knockout of Mcl-1 are normal, while they are severely neutropenic (Dzhagalov et al., 2007), indicating that levels of this protein do not play a major role in their cell survival. Therefore, this result suggests that there are compensating mechanisms involving Bcl-2 or Bcl-X_L expression in purvalanol A treated PBMCs.

Neutrophil apoptosis induced by purvalanol A was investigated further using powerful neutrophil survival factor, GM-CSF, in order to determine if the effect of purvalanol A could override anti-apoptotic signal from GM-CSF. It was found that effects of purvalanol A on neutrophil apoptosis and Mcl-1 expression was partially blocked with GM-CSF as there was no significant difference when comparing the effects of purvalanol A alone and the effects of purvalanol A with GM-CSF. This relates to another study using roscovitine treatment in neutrophils, where it was reported that roscovotine induced apoptosis in neutrophils by overriding the effects of GM-CSF (Rossi et al., 2006).

These observations might suggest that purvalanol A has a specific effect on Mcl-1 expression in neutrophils. Mcl-1 protein is normally expressed in neutrophils and its level decrease before the onset of apoptosis (Edwards et al., 2004). It has been found that GM-CSF can increase Mcl-1 stability in neutrophils in order to delay apoptosis (Derouet et al., 2004). The experiments reported in this Chapter show that purvalanol A increased Mcl-1 turnover in neutrophils, as it does in KCL-22 cells. This supports the concept that Mcl-1 half-life can be decreased in the presence of certain apoptosis-inducing agonists, or enhanced by agents that delay apoptosis, such as GM-CSF (Derouet et al., 2006).

It was then necessary to investigate the effects of purvalanol A on several important signalling pathways in neutrophils. It was found that purvalanol A resulted in a significant up-regulation of phosphorylated Akt and p-38 while GM-CSF resulted in a significant up-regulation of phosphorylated Erk, STAT3, and Akt. This supports another study that used GM-CSF in neutrophils (Suzuki et al., 1999, Nolan et al., 1999, Al-Shami et al., 1998). However, the results obtained in this Chapter showed that purvalanol A increased the level of Phospho-p38 in neutrophils. It has been demonstrated that cellular stresses can stimulate p38 MAPK activity to induce apoptosis (Aoshiba et al., 1999). The findings presented here support the notion that activated p38 generates a death signal in neutrophils (Frasch et al., 1998), perhaps directly or indirectly leading to increased turnover of Mcl-1. In addition, p38 MAPK inhibitor (BIRB796) can block the effect of purvalanol A in decreasing Mcl-1 expression in neutrophils.

The experiments described here provide preliminary results to show that purvalanol A induced apoptosis in neutrophils, as well as CML cell lines. Therefore, further experiments concerning drug toxicity are required in the next step of this study.

CHAPTER 7: EFFECTS OF JAK3 INHIBITOR, ZM39923, ON IMATINIB-SENSITIVE AND -INSENSITIVE CML CELL LINES

7.1 Introduction

The BCR-ABL tyrosine kinase contains several domains which promote protein-protein interactions. BCR-ABL can also serve as a domain for various adaptor proteins, such as GRB2, leading to protein phosphorylation. As a consequence, many signalling pathways are activated including, RAS, PI3K, and JAK-STAT (Deininger et al., 2000, Danial and Rothman, 2000). These activated signalling pathways can regulate various cellular effects such as cell proliferation and suppression of apoptosis, which are implicated in CML pathology (Ren, 2005).

The Janus family of kinases (JAKs) are crucial signal transducers for the receptors of several cytokines, growth factors, and interferons which intrinsically lack of kinase activities, such as the receptors for erythropoietin (Epo), granulocyte-macrophage stimulating factor (GM-CSF), and interleukins (IL) (Schindler, 2002). The Janus family of kinases has four members including JAK1, JAK2, JAK3, and non-receptor tyrosine kinase 2 (TYK2). JAK1 is important in pro-inflammatory cytokine signalling such as IL-1, IL-6, and Tumour necrosis factor alpha (TNFα) (Ihle and Kerr, 1995). JAK2 plays a crucial role in hematopoietic growth factors signalling including GM-CSF, Epo and IL-3, while JAK3 is primarily expressed in haematopoietic cells (Ihle and Kerr, 1995). It has been reported that mice deficient in JAK3 developed severe combined immunodeficiency (SCID) (Nosaka et al., 1995) and TYK2 can transduce intracellular signals of cytokine receptors, such as IL-12 (Pesu et al., 2008).

JAKs can recruit the signal transducer and activator of transcription family (STATs) by SH2 domain recognition (Chen et al., 2003). JAKs activates STAT transcriptional activity, leading to the expression of genes involved in cell proliferation, apoptosis, differentiation, as well as the expression of inflammatory proteins (Figure 7.1) (Yu et al., 2009). JAK/STAT signalling pathways are critical in promoting the growth and survival of haematopoietic cells (Park et al., 1995).

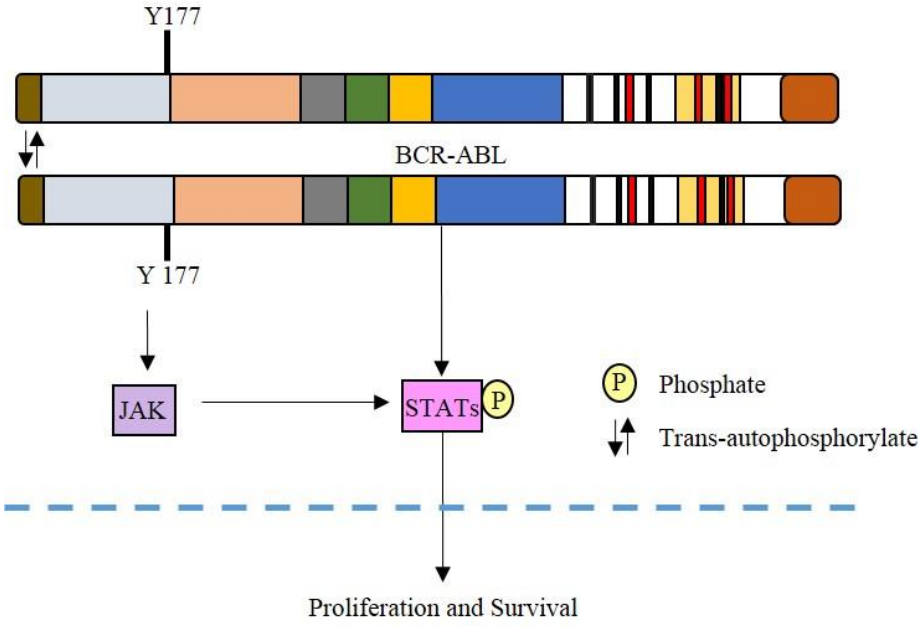

Figure 7.1 Effects of BCR-ABL on JAK/STAT signalling pathways. This figure shows how transcription factors, STATs are activated by BCR-ABL through direct phosphorylation or indirectly through phosphorylation by JAK (Redrawn from (Danial and Rothman, 2000)).

Therefore, several pharmacologic inhibitors that target JAK-STAT pathways have been developed and tested in clinical trials in recent years (Luo and Laaja, 2004). As JAK/STAT can act as a regulator of B-lymphocyte differentiation, it is a pharmaceutical target for several immune and autoimmune diseases such as type I diabetes, inflammatory bowel disease and rheumatoid arthritis (O'Shea et al., 2004). In addition, JAK/STAT inhibition was found to prolong allograft survival by targeting of JAK3 to inhibit immune rejection (Stepkowski et al., 2002).

Apart from inhibition of JAK/STAT in immune and autoimmune diseases, the inhibition of JAK/STAT represents a strategy for chemotherapeutic development (Quintas-Cardama et al., 2011). JAKs can be potential targets in cancer treatment due to the fact that many tumours exhibit constitutive STATs activation, including CML (Fabbro, 2012). STATs are constitutively activated in Philadelphia chromosome positive cells and previous studies have shown that STAT5 is a major regulator of CML cell proliferation while STAT1 is activated in some cases (Danial and Rothman, 2000). STATs also induce gene transcription of several Bcl-2 family proteins, such as the anti-apoptotic proteins, Bcl-X_L and Mcl-1. Bcl-X_L may be regulated by JAK/STAT signalling and the anti-apoptotic characteristics of CML cells (Danial and Rothman, 2000), while Mcl-1 has been reported to be transcriptionally controlled by STAT3 (Dong et al., 2011).

However, the point mutation of JAK2, V615F, has been identified recently in many types of myeloproliferative neoplasms (MPNs) (Quintas-Cardama, 2013). Thus, this mutation is considered to be a new therapeutic target. Roxutinib, the ATP competitive JAK2 inhibitor, has been developed and has now entered phase III clinical trials for the treatment of myelofibrosis. This inhibitor successfully decreased spleen size and improved symptoms related to haematological disease (Verstovsek et al., 2010).

Several small molecules which inhibit JAKs have been identified by high throughput screening methods in the last decade (Sonbol et al., 2013). Among these, ZM39923 displays distinct JAK inhibition. ZM39923 is a naphthyl ketone with an N-benzyl-N-isopropyl substitution which leads to superior JAK3 inhibition. It also inhibits JAK1, but is less potent compared to its effects on JAK3 (Brown et al., 2000). However, this inhibitor has not been studied in cancer cell lines, but the potent and selective inhibition of JAK3 might induce apoptosis in CML cells especially when used in combination with imatinib.

The aims of the work described in this Chapter were:

i) To determine the effects of the JAK3 inhibitor, ZM39923, on the growth and apoptosis of LAMA-84 and KCL-22 cell lines.

ii) To measure the expression of anti-apoptotic protein, Mcl-1, in these two cell lines and to determine how their protein levels are affected by ZM39923.

7.2 Methods

ZM39923 was used at a range of concentrations between 10, 30, and 60 µM, unless stated otherwise, which was shown in previous studies in this laboratory (Davies, 2013, Jackson, 2014) to induce effects on the growth of LAMA-84 and KCL-22 cell lines. As ZM39923 was dissolved in DMSO, all control experiments contained equivalent amounts of this solvent. In experiments using ZM39923 plus other compounds dissolved in DMSO, the maximal DMSO used in experiments was 0.7 % (v/v) and this concentration did not have any measured effect of any of the cell parameters analysed.

7.3 Results

Initial experiments measured the effects of ZM39923 and imatinib on apoptosis of these cell lines using the Viacount assay and the cell cycle assay described in Chapter 2. The levels of expression of anti-apoptotic protein, Mcl-1 were also measured by western blot.

7.3.1 Effects of ZM39923 on the viability of LAMA-84 and KCL-22 cell lines

Previous work in this laboratory (Davies, 2013, Jackson, 2014) showed that ZM39923 caused a decrease in cell viability in both cell lines. Therefore, it was important to investigate the effects of ZM39923 on cell viability further in these cell lines. LAMA-84 and KCL-22 were either incubated with a range of concentrations of 10, 30, or 60 µM ZM39923 alone or in combination with 10 µM imatinib for 24 h. Then, cell viability was determined by flow cytometry using the Viacount assay.

Figure 7.2 confirmed previous results in Chapter 3 that imatinib decreased the viability of LAMA-84 cells significantly ($n=3$, $p\leq0.01$), but not KCL-22 cells ($n=3$, $p>0.05$) under these experimental conditions. In the LAMA-84 cell line, viability was also decreased following ZM39923 treatment in a dose-dependent manner. At the lowest concentration used of ZM39923 (10 µM), the viability was decreased significantly ($n=3$, $p\leq0.01$) from 92.2 ± 2.3 % in untreated controls to 78.2 ± 1.3 % in ZM39923 treated samples. In addition, viability was further decreased following ZM39923 treatment in combination with imatinib (from 57.5 ± 3.3 % and 78.2 ± 1 % in 10 µM imatinib and ZM39923 treated sample, respectively compared to 38.3 ± 4.2 % in 10 µM ZM39923 plus imatinib treated sample). This difference between both imatinib and ZM39923, used alone, and ZM39923 together with imatinib was found to be significance ($n=3$, $p\leq0.05$). Thus, the combined effects of these two drugs was greater than when either drug was used alone.

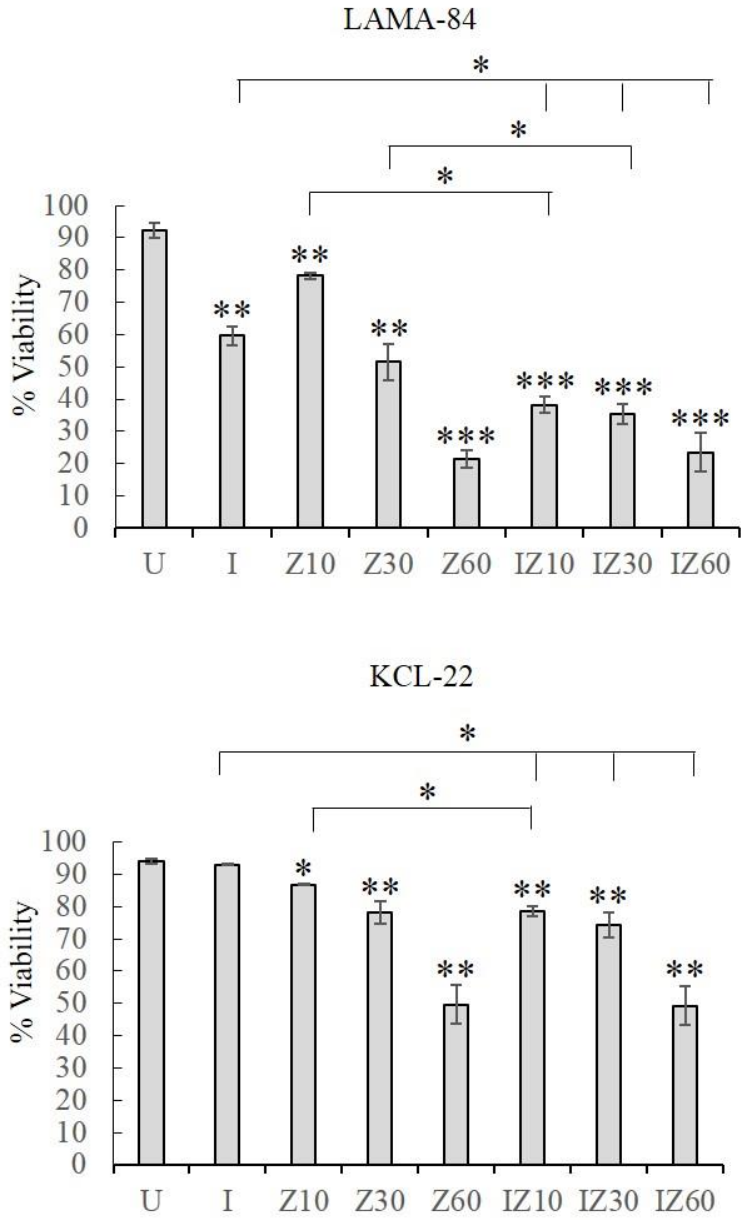

Figure 7.2 Differential sensitivity of LAMA-84 and KCL-22 cell lines to imatinib and ZM39923. Both cell lines were incubated in an absence (UT) and presence of 10 µM imatinib (I) or 10, 30 or 60 µM ZM39923 (Z) alone or in combination (IZ) for 24 h. Viability was determined using the Viacount assay. Data are shown as mean (±SEM, n=3) *= $p \leq 0.05$, **=$p \leq 0.01$, ***=$p \leq 0.001$ compared to untreated controls (paired two-tailed student's t-test).

Similar to LAMA-84, ZM39923, used alone, decreased KCL-22 cell viability significantly ($n=3$, $p \leq 0.05$) in a dose-dependent manner (from 94.2 ± 0.8 % in untreated control to 82.1 ± 0.2 % in 10 µM ZM39923 treated sample). However, decreases in viability were lower than those observed in LAMA-84 cells. Also, the combination of ZM39923 with imatinib decreased viability greater than when both imatinib and ZM39923 were used alone (from 93 ± 0.2 % and 86.8 ± 0.2 % in 10 µM imatinib and ZM39923 treated sample, respectively to 78.5 ± 1.6 % in 10 µM ZM39923 and imatinib treated sample). These differences were found to be significant ($n=3$, $p \leq 0.05$). Again, the effects of both drugs used together were greater than the effects observed using either drug alone.

7.3.2 Effects of ZM39923 on the cell cycle kinetics of LAMA-84 and KCL-22 cell lines

The results from the Viacount assay, showed that ZM39923 decreased viability in both LAMA-84 and KCL-22 cell lines significantly. Therefore, the effects of ZM39923 were determined on the cell cycle kinetics in order to confirm the previous results from Viacount assay. Both cell lines were incubated with imatinib (10µM), ZM39923 (10, 30 and 60 µM) or a combination of both for 24 h and cell cycle kinetics were analysed using flow cytometry.

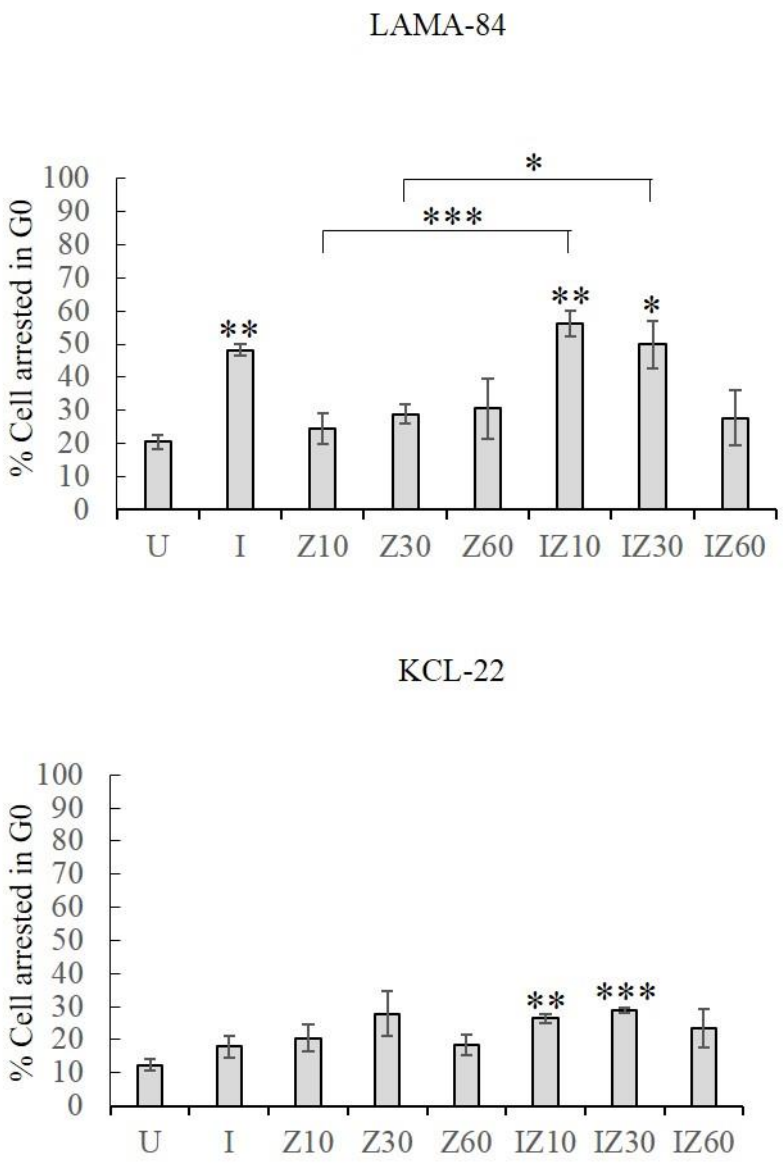

Figure 7.3 Effect of imatinib and ZM39923 on LAMA-84 and KCL-22 cell cycle kinetics. Cells were incubated as described in the legend to Figure 7.2. Cell cycle parameters (expressed as a percentage of the total cell population) of cells arrested in G0 were determined by measuring DNA content and flow cytometry. Data are shown as mean (±SEM, n=3) *=p≤0.05, **=p≤0.01, ***=p≤0.001 compared to untreated controls (paired two-tailed student's t-test).

After incubation of LAMA-84 cells with imatinib, the percentage of cells arrested in G0 increased significantly (n=3, $p \leq 0.01$), as seen previously in Chapter 3. ZM39923 slightly increased the percentage of cells arrested in G0, with corresponding slight decreases in cells in all other phases (results not shown) in dose-dependent manner (Figure 7.3). However, cell viability measured by Viacount assay was decreased significantly by ZM39923 and so a greater effect on accumulation of cells in G0 might have been predicted. There was a significant difference (n=3, $p \leq 0.01$) in the number of cell arrested in G0 following incubation with 10 µM and 30 µM ZM39923 alone and in combination with imatinib (from 24.5 ± 4.7 % in 10 µM ZM39923 treated samples to 56.2 ± 3.8 % in 10 µM ZM39923 plus imatinib treated sample), but the numbers of cells arrested in G0 was not different compared to imatinib, used alone, i.e. there was no increase in G0 arrest in cells treated with the combination of drugs, compared to the effects of imatinib alone.

In KCL-22 cells, there was an increase of the number of cells arrested in G0 in dose-dependent manner following ZM39923 incubation, but this increase did not reach statistical significance (n=3, $p > 0.05$). However, at 10 µM and 30 µM ZM39923 the number of cells arrested in G0 was significantly increased (n=3, $p \leq 0.01$ and $p \leq 0.001$, respectively) when used in combination with imatinib (from 12.35 ± 1.67 % in untreated control to 26.3 ± 1.4 % in 10 µM ZM39923 and imatinib treated sample).

7.3.3 Effects of ZM39923 on Mcl-1 expression in LAMA-84 and KCL-22 cell lines

As Mcl-1 is a major anti-apoptotic protein that regulates apoptosis in LAMA-84 and KCL-22 cells (details in Chapter 3, 4 and 5). Thus, it is crucial to determine the effects of ZM39923 on the expression of Mcl-1 in these cells. Both cell lines were incubated with 10μM imatinib, 10, 30 or 60 μM ZM39923 or both for 24 h. After incubation, the levels of expression of Mcl-1 were determined using western blotting.

In LAMA-84 cells, treatment with imatinib decreased the expression of Mcl-1 significantly (n=3, $p \leq 0.01$) (Figure 7.4). This confirms the resulted obtained in previous Chapters. ZM39923, used alone at 30 and 60 μM caused a slight decrease in Mcl-1 expression in a dose-dependent manner, but this did not reach statistical significance (n=3, $p > 0.05$). In contrast, the combination of ZM39923 with imatinib significantly decreased Mcl-1 expression (n=3, $p \leq 0.05$). This dramatic decrease in Mcl-1 expression is different from the effects of ZM39923 used alone (n=3, $p \leq 0.05$), this was a significant difference when compared with imatinib as a single treatment (from 108.2 ± 7.2 % in 10 μM ZM39923 treated sample to 28.5 ± 8.5 % in 10 μM ZM39923 plus imatinib treated sample, (n=3, $p \leq 0.05$).

In KCL-22 cells, imatinib did not decrease Mcl-1 expression confirming the results from previous Chapters. In the same way with LAMA-84 cells, 30 μM and 60 μM ZM39923, used alone caused only a slight decrease in Mcl-1 expression in a dose-dependent manner (n=3, $p > 0.05$). The combination of 30 μM and 60 μM ZM 39923 with imatinib decreased Mcl-1 expression significantly (n=3, $p \leq 0.05$). ZM39923, used together with imatinib caused significant decreases in protein levels (n=3, $p \leq 0.05$) when compared to ZM39923 and imatinib as single agents (from 99.6 ± 13.6 % and 93.8 ± 7.7 % in 10 μM imatinib and 30 μM ZM39923 treated samples, respectively to 35.2 ± 4.7 % in 30 μM ZM39923 plus imatinib treated sample) (Figure 7.5).

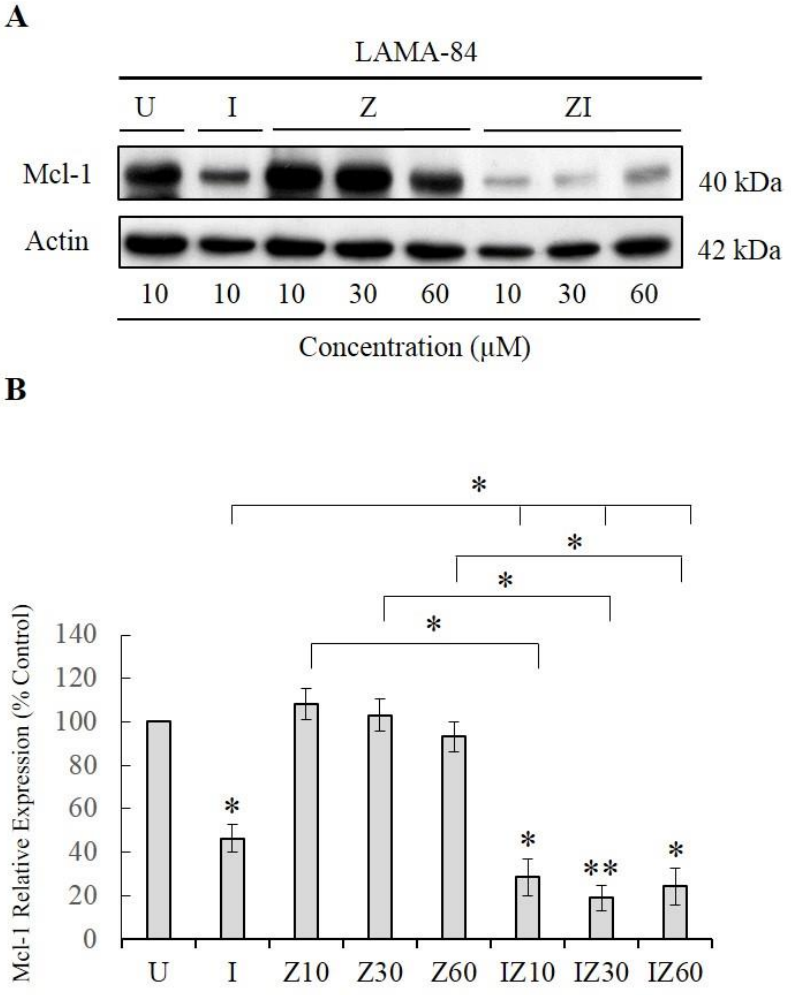

Figure 7.4 Effects of imatinib and ZM39923 on Mcl-1 protein expression in LAMA-84 cell line. Cells were incubated as described in the legend to Figure 7.2. **(A)** Levels of Mcl-1 expression were measured by western blotting using an anti-Mcl-1 antibody. **(B)** Densitometric analysis of Mcl-1 relative expression. Data expressed as a % of untreated samples (±SEM, n=3), * = $p \leq 0.05$, ** = $p \leq 0.01$ (paired two-tailed student's t-test). Western blot shown is representative from n=3 experiments.

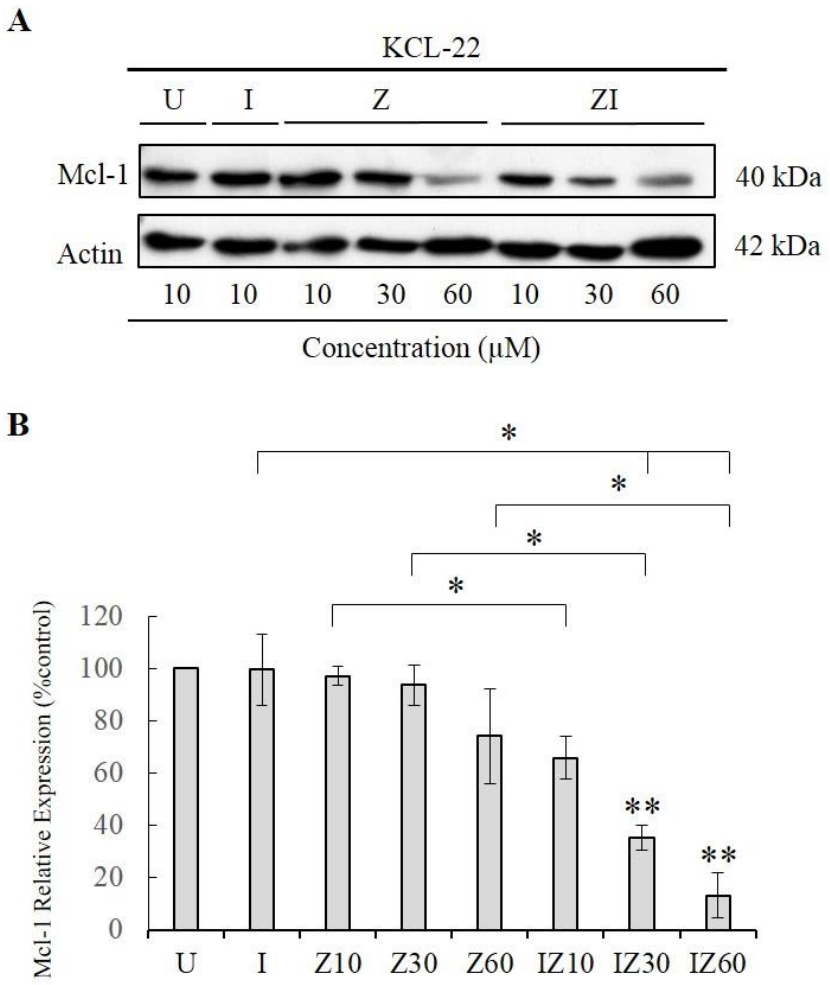

Figure 7.5 Effects of imatinib and ZM39923 on Mcl-1 protein expression in KCL-22 cell line. Cells were incubated as described in the legend to Figure 7.2. **(A)** Levels of Mcl-1 expression were measured by western blotting using an anti-Mcl-1 antibody. **(B)** Densitometric analysis of Mcl-1 relative expression. Data expressed as a % of untreated samples (±SEM, n=3), * = $p \leq 0.05$, ** = $p \leq 0.01$ (paired two-tailed student's t-test). Western blot shown is representative from n=3 experiments.

7.3.4 The time course of effects of ZM39923 and imatinib on Mcl-1 expression

As shown above, 30 µM ZM39923 together with 10 µM imatinib significantly decreased Mcl-1 expression levels in both LAMA-84 and KCL-22 cell lines, following 24 h incubation ($n=3$, $p \leq 0.01$). Therefore, the effects on Mcl-1 expression of this drug combination were determined in time course analysis. Both cell lines were incubated in the absence (untreated control), or with 10µM imatinib, 30µM ZM39923, and both drugs together for 0, 3, and 6 h. After incubation, cell lysates were prepared and Mcl-1 expression levels were analysed using western blotting.

In LAMA-84 cell line, Mcl-1 expression remained fairly constant throughout the 6 h of incubation with ZM39923, imatinib, and both ZM39923 and imatinib together (Figure 7.6A). However, while 30 µM ZM39923 together with imatinib decreased Mcl-1 expression dramatically to less than 20 % after 24 h incubation (Figure 7.3A), this combination did not affect Mcl-1 expression within this 6 h time course incubation.

Similar to LAMA-84 cells, the combination of ZM39923 and imatinib did not decrease Mcl-1 expression in KCL-22 cell over the period of the 6 h incubation. In contrast, there was a decrease in Mcl-1 expression when cells were incubated with ZM39923 alone (Figure 7.6B).

To conclude, Mcl-1 expression levels did not decrease upon exposure to ZM39923 together with imatinib treatment, in both cell lines over the 0-6 h time incubation period. However, over a 24 h incubation time, this combination caused significant decreases in Mcl-1 levels in both cell lines ($n=3$, $p \leq 0.01$).

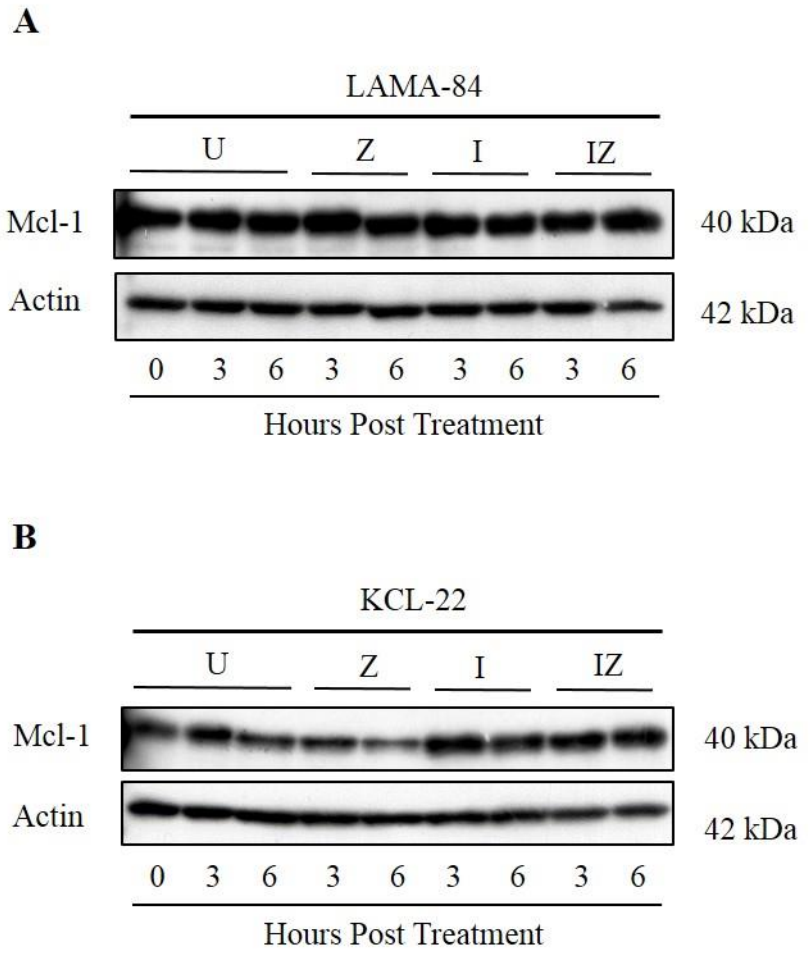

Figure 7.6 Effects of ZM39923 and imatinib on Mcl-1 expression in LAMA-84 (A) and KCL-22 (B) cell lines over an incubation period of 6 h. Cells were incubated in an absence (U) or presence of 30 µM ZM39923 (Z), 10 µM imatinib (I), or both (ZI) and lysates were prepared at 0, 3, and 6 h. Levels of Mcl-1 expression were measured by western blotting using an anti-Mcl-1 antibody (n=1).

7.3.5 The effect of ZM39923 and imatinib on the half-life of Mcl-1

The following experiments were performed to determine if the combination of ZM39923 and imatinib caused apoptosis via changes in the turnover rate of Mcl-1 in both cell lines.

LAMA-84 and KCL-22 cell lines were incubated with CHX, prior to ZM39923 and imatinib treatment. After this pre-incubation period, the cycloheximide-treated cells were incubated for 1, 2, 4, and 6 h in the absence and presence of 10 μM ZM39923, 10 μM imatinib, or both drugs together. After incubation, protein extracts were prepared at the indicated time points. Levels of Mcl-1 protein in these cells that were blocked in protein synthesis were determined by western blotting.

In LAMA-84 cells, the half-life of Mcl-1 in untreated controls was around 2 h (Figure 7.6). However, ZM39923, imatinib, and when both drugs were used together, resulted in an increase of the half-life of Mcl-1 to more than 6 h (Figure 7.6). This increased stability of Mcl-1 was greatest when the two drugs were used in combination.

Similarly, ZM39923 and imatinib, used alone, increased the half-life of Mcl-1 from around 2-3 h in untreated control to around 6 h in KCL-22 cells. However, the combination of ZM39923 and imatinib did not increase the half-life of Mcl-1 and this combination caused no change in half-life of Mcl-1 when compared to untreated control (around 2-3 h).

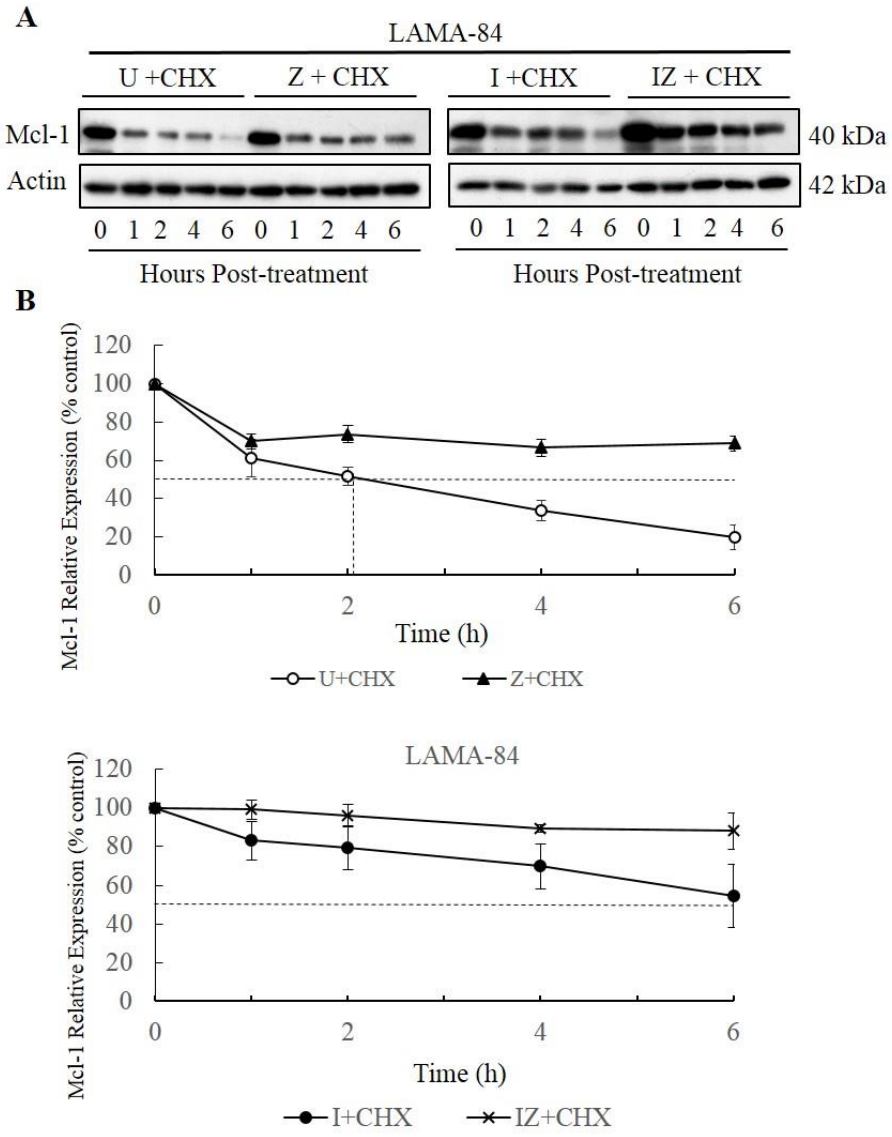

Figure 7.6 Effect of ZM39923 and imatinib on the half-life of Mcl-1 in LAMA-84 cells. Cells were pre-incubated for 10 min with cycloheximide (CHX) prior to 0, 1, 2, 4, and 6 h incubation in absence (U), or presence of 30 μM purvalanol A (P). **(A)** Shows a typical western blot analysis, while **(B)** shows densitometric analysis. The half-life were indicated with IC_{50} (shown in dashes). Data are expressed as % of untreated samples (±SEM, n=3). Western blot shown is representative from n=3 experiments

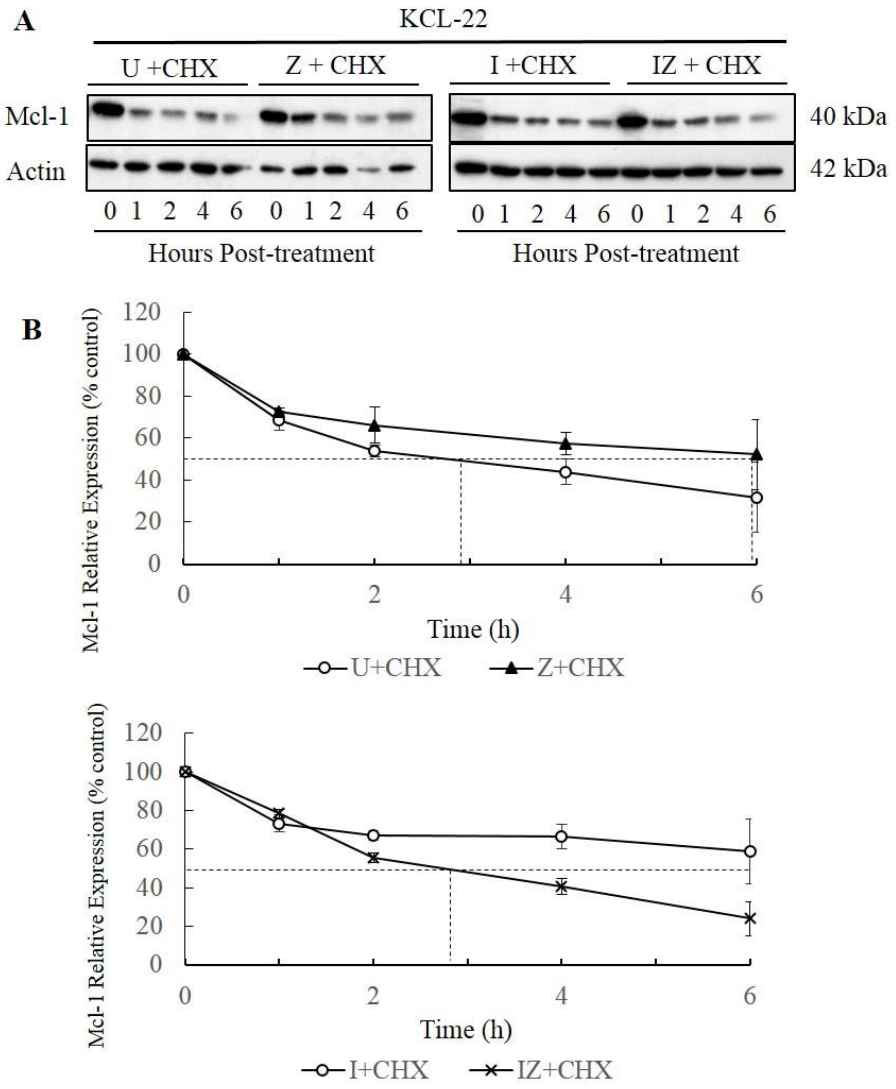

Figure 7.7 Effect of ZM39923 and imatinib on the half-life of Mcl-1 in KCL-22 cells. Cells were pre-incubated for 10 min with cycloheximide (CHX) prior to 0, 1, 2, 4, and 6 h incubation in absence (U), or presence of 30 μM purvalanol A (P). **(A)** Shows a typical western blot analysis, while **(B)** shows densitometric analysis. The half-life were indicated with IC_{50} (shown in dashes). Data are expressed as % of untreated samples (±SEM, n=3). Western blot shown is representative from n=3 experiments.

7.4 Discussion and Conclusions

In this Chapter, the effects of ZM39923 were investigated in order to determine if this drug could be considered as an alternative therapeutic strategies to overcome TKI resistance and also to compare its effects with purvalanol A in order to understand the mechanisms of action.

Until now, there are no reports of the effects of the JAK3-specific inhibitor, ZM39923, in any CML cell line. Thus, this study provides the first preliminary results into the effects of ZM39923 on imatinib-sensitive and –insensitive CML cell lines. It was found that a low concentration of ZM39923 (10 μM) decreased cell viability in LAMA-84 and KCL-22 cells. However, this was not confirmed by cell cycle analysis. Interestingly, the use of a combination of ZM39923 and imatinib resulted in an enhanced response seen both in the cell viability and cell cycle results. These result supports the concept of combinatorial CML treatment by targeting other key signalling proteins alongside targetting TKIs. This finding supports several studies using inhibitors that block BCR-ABL downstream signalling pathways in combination with imatinib, which have found synergistic effects (Tipping et al., 2002, Radujkovic et al., 2005, Tseng et al., 2005, Kancha et al., 2008). For example, the combination of imatinib and PDK1 inhibitor (Tseng et al., 2005) and small molecule Grb2 SH2 domain binding antagonists, TB03, (Zhang et al., 2014) have been shown to induce synergistic effects in CML cell lines. In addition, an apoptosis induction in K562 cell line by TB03 was associated with cell cycle arrest. TB03 increased the number of cells arrested in G0/G1, with corresponding lower numbers of cells arrested in S/G2 phase of the cell cycle (Zhang et al., 2014). As ZM39923 sensitised KCL-22 cells to the effects of imatinib, this opens the possibility of consideration of a dual therapy option. This could decrease adverse effects from therapeutic agents that target processes in non-malignant cells as low concentrations of drug could be used in dual therapy.

These findings confirm the idea that JAK3 can be a therapeutic target for CML. One research group reported that JAK3 is physically associated with ABL by co-immunoprecipitation. They found that selective JAK inhibition decreased ABL kinase activity by 61 % at 6 h of treatment. This indicates that JAK3 is involved in ABL kinase constitutive activation (Lin et al, 2005). As a consequence, further investigation of the effects of ZM39923 on CML cell lines should improve molecular understanding and lead to more effective treatment of CML.

The effect of ZM39923 on the apoptotic protein, Mcl-1 was also determined in this Chapter as Mcl-1 is crucial in the regulation of apoptosis in CML (Aichberger et al., 2005). The results obtained show that ZM39923 in combination with imatinib, decreased level of Mcl-1 expression significantly over 24 h of incubation in both cell lines. It has been reported that Mcl-1 expression was decreased by another JAK3 inhibitor, PF956580, in chronic lymphocytic leukaemia cells (Steele et al., 2010).

However, this decrease in Mcl-1 expression did not occur within 6 h incubation time (from time course analysis experiment). Also, ZM39923 used alone and ZM39923 used with imatinib did not decrease the half-life of Mcl-1 protein in both cell lines. Therefore, the decrease in Mcl-1 caused by the combination of ZM39923 and imatinib is not due to any changes in the rate of protein turnover (as for purvalanol A induced Mcl-1 turnover in KCL-22 cells), but therefore more likely to be due to changes in transcription/translation of the protein.

In conclusion, ZM39923 was demonstrated in this Chapter to decrease cell viability in LAMA-84 and KCL-22 cell lines and decrease in Mcl-1 expression levels, when used in combination with imatinib. These observations indicate that it induces apoptosis and sensitises imatinib-resistant cells to treatment with imatinib. This work should promote more studies regarding the use JAK3 inhibitors in the treatment of CML. However, the mechanism of action of this inhibitor on Mcl-1 expression is still unclear and requires further investigations beyond these preliminary results.

CHAPTER 8: GENERAL DISCUSSION AND CONCLUSIONS

Chronic Myeloid Leukemia is associated with a single genetic abnormality known as the Philadelphia (Ph) chromosome. This chromosome results from a t(9;22) reciprocal translocation which results in the formation of the BCR/ABL fusion gene (Deininger et al., 2000). This gene encodes a constitutively-active protein tyrosine kinase, leading to a de-regulation of tyrosine kinase activity in the cells (Quintas-Cardama and Cortes, 2009). A major breakthrough in the treatment of CML was the development of the specific BCR-ABL inhibitor, imatinib, which is now used as the first line therapy. Following its initial success, resistance to this drug developed via several mechanisms, including mutations in the BCR-ABL gene (Corbin et al., 2003). Hence, new generations of tyrosine kinase inhibitors (TKIs), for example, nilotinib, dasatinib, and ponatinib have been developed.

While these new generation tyrosine kinase inhibitors have overcome many of the problems associated with failure of patients to respond to imatinib because of mutations, resistance to these inhibitors is still a problem. This is because during the advanced stages of CML, rates of mutations are high (Zhang et al., 2009). In addition, the BCR-ABL gene may become amplified and other mechanisms of resistance (e.g. changes in activity of drug influx and efflux mechanisms) may develop (Mahon et al., 2000). Therefore, there is a continued need to find new ways to induce CML cell death, perhaps when used in combination with TKIs. Inhibitors of other target kinases may therefore have clinical potential. Among these kinases, CDK inhibitors have shown promising results.

CDKs are a family of serine/threonine kinases that are involved in cell cycle progression (Diallo and Prigent, 2011), expressed in both unicellular and multicellular organisms (Malumbres and Barbacid, 2005). In several pathological conditions, there are abnormalities in CDK expression and regulation such as viral infections, neurodegenerative disorders and cancer (Cicenas et al., 2014). Therefore, several small molecule CDK inhibitors have been developed in order to treat these diseases.

Most recently (in February 2015), the CDK4 and 6 inhibitor, pablociclib, was approved by the FDA to treat breast cancer (Turner et al., 2015). In addition, several CDK inhibitors have been developed in both preclinical and clinical studies and most of these inhibitors have shown promising results and now in the process of drug development (reviewed in (Cicenas et al., 2014)). It has been reported that CML is associated with altered CDK activity. For example, a deregulated oncogene, ras, targets CDKs leading to their inappropriate activation. Therefore, CDK inhibitors might be a promising alternative CML treatment (Cortez et al., 1997).

Recently, the combination use of flavopiridal and imatinib has begun in phase I clinical trials in CML patients and is encouraging responses have been observed, particularly in imatnib-resistant CML patients (Bose et al., 2012). This follows from experiments that demonstrated its ability to induce apoptosis in human leukaemic cells in both *in vitro* and in *vivo* experiments (Decker et al., 2001). In addition, it was found to enhance the activity of imatinib in imatinib-insensitive CML cell lines (Yu et al., 2002).

As a result, CDK inhibitors, used alone and in combination with TKIs, are now a promising avenue for treatment of imatinib-resistant CML. Therefore, the main objective of this study was to investigate the effects of CDK inhibitor, purvalanol A, on imatinib-sensitive and –insensitive chronic myeloid leukeamia cell lines. In addition, the effects of purvalanol A were also investigated in normal white blood cells, including neutrophils and PBMCs to provide the first experiments to evaluate its toxicity. Lastly, the

effects of other inhibitor, ZM39923 (JAK3 inhibitor), on imatinib-sensitive and –insensitive chronic myeloid leukeamia cell lines were also investigated to understand the potential of JAK inhibition as a therapeutic approach for CML.

The main findings of my studies were as follows:

i) Imatinib induced apoptosis in imatnib-sensitive CML cell line (LAMA-84), but not imatinib-insensitive CML cell line (KCL-22).

ii) Purvalanol A induced apoptosis in both LAMA-84 and KCL-22 cells.

iii) Purvalanol A induced mitochondrial depolarization in both cell lines within 2 h of incubation.

iv) Purvalanol A increased Mcl-1 (both protein and mRNA) turnover in KCL-22 cells, but not in LAMA-84 cells.

v) Purvalanol A induced apoptosis in neutrophils, but not PBMCs.

vi) Purvalanol A resulted an increase of phosphorylated p38 MAPK in KCL-22 cells and neutrophils.

vii) JAK inhibition induced apoptosis in both CML cell lines when used in combination with imatinib.

Results from this study indicate, for the first time, that the Cdk2 inhibitor, purvalanol A can induce rapid cell death in an imatinib-insensitive cell line, KCL-22. In spite of its known effects on Cdk2 and cell cycle arrest, the effects observed on KCL-22 viability are likely to be independent of its effects on the cell cycle. Instead, a clear effect of this inhibitor was demonstrated on expression of the anti-apoptotic protein, Mcl-1 in the absence of any detectable changes in expression of other anti-apoptotic proteins, such as Bcl-2 and Bcl-X_L. While purvalanol A decreases mRNA levels within 30 min incubation, profound and rapid decreases in protein levels were observed that are attributable to a decrease in the half-life of this protein, from around 3 h to around 1.5 h. This decrease in Mcl-1 stability, together with the decrease in mRNA levels, resulted in a marked decreased expression of this protein that was significant by 1 h treatment with this drug. Thus, this rapid decline in Mcl-1 levels is probably responsible for the rapid activation of apoptosis in these cells. In contrast to purvalanol A, the JAK3 inhibitor, ZM39923, did not increase Mcl-1 turnover, while it induced apoptosis in both cell lines.

Mcl-1 is an unusual member of the Bcl-2 family and has properties that distinguish it from other family members (Reynolds et al., 1994). It is a much larger protein (37 kDa) than either Bcl-2 or Bcl-X_L (22 and 25 kDa, respectively) and while it shares some homology in its BH domains, it possesses a unique N-terminal domain that is responsible for its special properties (Kozopas et al., 1993). This N-terminal domain contains PEST domains and other motifs that are subject to reversible post-translational modifications, particularly phosphorylation at Serine and Threonine residues (Thomas et al., 2010) that alter and regulate its function. The protein has a very short half-life (2-3 h depending on the cell type) and this half-life can be shortened or extended by phosphorylation on key residues to either accelerate or delay, respectively, cell apoptosis (Thomas et al., 2012). In human neutrophils and in some cancers, apoptosis delaying agents have been shown to extend Mcl-1 half-life, whilst other agonists (e.g. sodium salicylate, phosphatase inhibitors) result in accelerated turnover of this protein (Derouet et al., 2006) with a concomitant enhancement of

apoptosis. In view of the rapid effects on Mcl-1 stability and turnover, purvalanol A could either directly or indirectly lead to a change in the phosphorylation status of Mcl-1 that regulates its stability. Many of the Serine/Threonine residues targeted and the kinase/phosphatase systems responsible for many of these functional changes have been identified (Kobayashi et al., 2007, Domina et al., 2004, Maurer et al., 2006, Inoshita et al., 2002) and it will now be necessary to identify the post-translational modifications of Mcl-1 that are directly or indirectly affected by purvalanol A.

While imatinib induced cell death in LAMA-84 cells via a caspase-dependent process, this drug had little effect on apoptosis in KCL-22 cells, although it did induce a partial accumulation of these cells in G1. Imatinib treatment of LAMA-84 cells resulted in a decrease in Mcl-1 protein levels (but not Bcl-2 or Bcl-X_L levels), both apoptosis and this decrease in Mcl-1 were prevented by caspase inhibition. This observation would appear to confirm the key role for Mcl-1 in imatinib-induced apoptosis of CML cells (Aichberger et al., 2005). However, this decrease in Mcl-1 levels in LAMA-84 cells was not associated by any detectable changes in Mcl-1 protein stability. It is therefore likely that imatinib negatively affects MCL1 transcription/translation, rather than inducing a post-translational modification that affect protein stability in these cells.

In LAMA-84 cells, while purvalanol A treatment had little effect on Mcl-1 protein levels, it resulted in a slight decrease in expression of Bcl-2, which (as in human neutrophils (Moulding et al., 2001)) is not expressed in KCL-22 cells. If purvalanol A does indeed affect the activity of a kinase/phosphatase responsible for induction of a post-translational modification of Mcl-1 to decrease its stability, it may be hypothesised that this kinase/phosphatase system is not activated or is absent in LAMA-84 cells. Therefore, the levels of activation of a number of key intracellular signalling systems that have been implicated in regulation of myeloid cell function and apoptosis in these two cell lines were investigated (Quintas-Cardama and Cortes, 2009). There were clear differences in the levels of activation of a number of these systems in these cells types and differences

in their responses to imatinib and purvalanol A. For example, pSTAT3 and pAkt could not be detected in LAMA-84 cells in spite of the fact that non-phosphorylated proteins appeared to be detected by the pan-antibodies used. These experiments also indicated that while imatinib had little effect on apoptosis in KCL-22, this drug completely abrogated activation of both pErk and pAkt in these cells, confirming a recent study (Ohmine et al., 2003). These signalling systems have previously been implicated in generating anti-apoptotic signals in myeloid cells (Quentmeier et al., 2011, Cortez et al., 1997) and yet knock-down by imatinib had little effect on apoptosis in KCL-22 cells. While imatinib had little effect on STAT3 activation in these cells, purvalanol A completely ablated activation of this signalling pathway. Whether STAT3 directly or indirectly regulates Mcl-1 stability is yet to be determined. Curiously, in KCL-22 cells (and to a lesser extent in LAMA-84 cells) purvalanol A induced a rapid and significant increase in activation of p38-MAPK. It has previously been proposed that this signalling molecule may generate death signals (Zarubin and Han, 2005, Koul et al., 2013, Jiang et al., 2014) and the present results lend some support to this notion. In this study, the effects of purvalanol A on CML cell lines were investigated further using the p-38 MAPK inhibitor, BIRB796, that can inhibit all isoforms of p38 MAPK (Kuma et al., 2005). The combination of BIRB796 and purvalanol A was found to decrease CML cell apoptosis and increase Mcl-1 expression in both CML cells and neutrophils compared to purvalanol A, used alone. This suggests p38 MAPK plays a role in the mechanism of action purvalanol A. It is possible that activated p38-MAPK can directly or indirectly result in a post-translational modification of Mcl-1 to increase its rate of turnover.

BCR/ABL has been reported to connect with several signalling pathways such as Erk, Akt, and JAK-STATs. Recently, p38 MAPK has also been reported to be modulated by BCR-ABL (Sanchez-Arevalo Lobo et al., 2005). p38 comprises of 4 isoforms, α, β, γ, and δ. These isoforms shares more than 60 % sequence homology (Wilson et al., 1996). p38 isoforms can be phosphorylated by MKK3, 4, and 6 to activate various substrates, including transcription factors and protein kinases. This leads to

inflammatory response and cell differentiation (Cuenda and Rousseau, 2007). Various agents including TKIs (Dumka et al., 2009), chlorogenic acid (Bandyopadhyay et al., 2004), and Ara-C (Sanchez-Arevalo Lobo et al., 2005) were found to be associated with the activation of p38 MAPK in order to induce apoptosis in CML cells. Therefore, this supports the finding that p38 MAPK is associated with the apoptosis induced by purvalanol A.

Recent studies in human cancer cell lines have shown that purvalanol A treatment inhibited both CDK activity and the expression of several anti-apoptotic proteins of the Bcl-2 family, such as Bcl-2 and Bcl-X_L (Iizuka et al., 2007, Iizuka et al., 2008). Purvalanol A was also found to interact with purified Cdk2 and this may explain its anti-mitotic properties (Knockaert et al., 2002a). It inhibits the phosphorylation of Cdk substrates, such as Retinoblastoma protein (Rb) and cyclin E. Moreover, it increases the level of the Cdk inhibitory protein, p21 $^{WAF1/CIP1}$. These effects lead to inhibition of cell proliferation in a range of human cancer cell lines and mouse fibroblasts (Villerbu et al., 2002). However, its actual intracellular targets are still undefined. One group has reported that purvalanol also interacts with p42/p44 MAPK protein in various cell lines, as detected by affinity chromatography (Knockaert et al., 2002b). In addition, purvalanol inhibited p42/p44 MAPK activities in dose-dependent manner in the Chinese hamster lung fibroblast cell line, CCL39 (Knockaert et al., 2002b). This suggests that the anti-proliferative effects of purvalanol may be mediated by both Cdk inhibition and interactions with p42/p44 MAPK. It has also been shown that purvalanol A suppresses c-Src transformation by inhibiting both cell cycle progression and c-Src signalling. Therefore, purvalanol A could be a potential inhibitor of cancers that are characterised by c-Src up-regulation (Hikita et al., 2010).

Purvalanol A has now been used in preclinical trials for treating several cancers (Cicenas and Valius, 2011, Cicenas et al., 2014) and so its toxicity, mechanism of action and effects on drug transporter processes purvalanol A are becoming characterised. Therefore, the effects of purvalanol A on neutrophils and PBMCs were investigated in this study to provide preliminary results regarding drug toxicity in primary leukocytes.

Purvalanol A was found to induce apoptosis in neutrophils, but not PBMCs. However, it resulted in a decrease in Mcl-1 expression in both neutrophils and PBMCs. In spite of the decrease in Mcl-1 expression, there was an elevated expression of Bcl-2 in purvalanol A treated PBMCs. These results indicate that purvalanol A might cause toxicity during long-term treatment as it induced apoptosis in normal white blood cells, particularly in neutrophils. Therefore, for this compound to be taken forward as a therapeutic option, further toxicity studies will be required to determine the therapeutic concentration that is safe to use and causes minimal side effects.

Future directions

Before entering advanced preclinical and clinical study, purvalanol A should undergo more extensive tests to determine if it is ready to administer in humans. In this study, preliminary results regarding effects of purvalanol A *in vitro* have been provided, but, *ex vivo*, and *in vivo* studies are also required to determine its mechanism of action and potential effects on humans.

Further studies should recruit CML patients in order to investigate whether purvalanol A could also induce cell death in primary CML cells *ex vivo*, as well as in CML cell lines. Primary cells can be isolated from CML patients following leukapheresis. Leukapheresis is performed in CML patients with high levels of white blood cells and this procedure leads to decreases in the number of white blood cells. (Ranganathan *et al.,* 2008). These white blood cells recovered by this process can be tested *ex vivo* and thus provide more data for preclinical studies.

Furthermore, *in vivo* studies are also important to provide more clinically-relevant data perhaps using using animal models. For example, rodent and non-rodent mammalian models can be used to determine pharmacokinetic profiles and toxicity patterns (Steinmetz and Spack, 2009).

Apart from concerns over the toxicity associated with long term use of drugs such as this, other areas of further investigation would be studies of signalling pathway activation in both CML cell lines and neutrophils. These could explain the mechanisms of purvalanol A induced apoptosis in these cells. In this study, p-38 MAPK was activated by purvalanol A in both KCL-22 cells and neutrophils. In addition, purvalanol A also increased Mcl-1 turnover in these cells, but not in LAMA-84 cells. This might suggest that the molecular phenotype of KCL-22 cells is close to that of mature neutrophils in terms of signalling pathways. BIRB796 could be further used to investigate the role of p38 MAPK in the mechanism of action purvalanol A and its role in Mcl-1 turnover on both CML cells and neutrophils. As described in this thesis, the preliminary experiments show encouraging results.

In this study, ZM39923 was demonstrated to decrease cell viability and Mcl-1 expression levels in both LAMA-84 and KCL-22 cell lines. The results obtained indicate that ZM39923 sensitises imatinib-insensitive cells to treatment with imatinib. This paves the way for a dual therapy option in CML. ZM39923 can be used at lower concentrations with imatinib, thereby decreasing the possibility of off-target or adverse toxicity effects. In contrast to purvalanol A, there is less preclinical data provided for ZM39923 and thus it is necessary to further investigate its effects and mechanism of action before considering clinical use.

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